A biochemical engineering study of lysozyme refolding

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A BIOCHEMICAL ENGINEERING STUDY OF LYSOZYME REFOLDING

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Abstract

This thesis reports biochemical engineering studies on the refolding of lysozyme. The strategy employed is to quantify where possible the effect of all environmental parameters on refolding, identify and characterise the rate limiting steps and using a reaction engineering model suggest an optimal reactor conformation and conditions. Lysozyme was chosen as the model protein as it is well characterised.

The effect of temperature, pH, dithiothreitol and guanidine hydrochloride concentration, type of denaturant and protein concentration on the refolding yield of lysozyme have been investigated. Using this data it has be shown that refolding can be approximated to a first order process. Aggregation has been followed at different concentrations of lysozyme and can be described as a second order process. The apparent rate constant for refolding of lysozyme was found to be 0.147 min^{-1} and the apparent rate constant for aggregation was found to be 3.3 $mgml^{-1}min^{-1}$.

Using these rate constants, a competitive model of refolding versus aggregation has been written. The experimental results agree well with the results predicted by the model. A selectivity term based on the two competitive reactions has been introduced. It has been shown that refolding yields can theoretically be enhanced by stepwise or continuous addition of denatured lysozyme. Denatured reduced lysozyme in 0.1M acetic acid follows the predicted results well. Denatured reduced lysozyme in 6M GuHCl and 0.15M DTT does not follow the predicted results well. This has been shown to be due to the increasing concentrations of both guanidine hydrogen chloride and dithiothreitol.

The model was used to investigate the effect of protein concentration and yield in the refolding tanks on the yield and efficiency of the entire refolding process. Based on the total equipment purchase cost it has been shown that for the refolding of lysozyme an optimal refolding concentration of 0.22 mg/ml exists. The two most important costs have been shown to be ultrafiltration and fermentation. It has been shown theoretically that the total purchase cost and subsequently the production cost of any refolding process can be reduced significantly by recycling aggregated material from the refolding tanks to the solubilisation tanks.

Finally, refolding was enhanced by the *in vitro* use of the *E.coli.* molecular chaperone, GroEL. GroEL significantly improves the yield of refolded lysozyme in the range 0.015 mg/ml to 0.2 mg/ml. The recovery and effectiveness thereafter of GroEL has been studied. GroEL was recovered

(>80%) using ultrafiltration membranes of a molecular weight cut-off of 30 kD. It has been shown that after five refolding experiments the effectiveness of GroEL as a refolding enhancer is unaffected by the recovery process.

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1. Introduction

When human insulin was first expressed in *Escherichia coli (E.coli)* during the 1970's, a new era in protein production began. It seemed as if the successful expression of rare proteins was guaranteed. Unfortunately, as the work progressed it was found that many recombinant proteins were deposited in the cell as insoluble aggregates known as inclusion bodies. This dampened the enthusiasm of many researchers and it was not until the mid 1980's when the demand for therapeutic proteins boomed that intensive research on inclusion bodies resumed. Various properties of inclusion bodies such as purity, size, density, state of oxidation of thiol groups and hardness have all been studied in detail (Taylor *et al.* (1986), Hartley and Kane (1988)). Most of these findings have been summarised in reviews (Marston (1986), Kane and Hartley (1988), Mitraki and King (1989), Schein (1989)).

Despite the protein being in an inactive form, inclusion bodies offer several process advantages over the production of soluble protein. The inclusion bodies are often 75% pure product and they can be separated via centrifugation or micro filtration from cell debris once the cells have been harvested and lysed. They also are insoluble and are protected from proteolytic attack (Lazdunski (1989)). Inclusion bodies allow the production of proteins that would be detrimental to the cell in their active soluble form. The major disadvantage of producing proteins via inclusion bodies is the low yields of active protein achieved during refolding. The low yield is due to the non-productive aggregation of refolding intermediates.

Over the last ten years considerable effort has been put into trying to elucidate the biophysical aspects of protein refolding. The factors which dictate the three-dimensional structure of the protein still evade us, despite the vast amount of research that has been carried out. We know that the structure of a protein is determined by its primary sequence and

environmental conditions, yet our ability to predict the structure of a protein from its primary sequence is very limited. Due to this lack of understanding refolding is still a very empirical process and no rational basis for designing a process to produce recombinant protein exists. Refolding has been shown to be a first order reaction but as the protein refolds it is subject to a competitive aggregation reaction which can be approximated to a second order reaction. This limits the concentrations at which folding can be accomplished industrially. At present large dilution tanks are used to overcome the problem and these tanks can often account for 75% of the capital cost of a refolding process (Datar *et al.* (1993)).

Using dilution significantly increases the process stream volume and leads to high costs in downstream processing. Ultrafiltration costs for concentrating tissue plasminogen activator prior to purification account for approximately a quarter of the total annual material costs (Datar *et al.*, (1993)). If proteins can be refolded at higher concentrations savings in both capital and operating costs can be achieved.

The aim of this thesis is to enhance the refolding of lysozyme, increasing the yield and concentration of refolded protein and reducing the volume to be processed downstream. The strategy employed is to propose a refolding mechanism, identify and characterise the rate limiting steps, quantify the effect of key environmental parameters and using reaction engineering models suggest optimal reactor conformations and conditions. The proposed model is a competitive model, refolding versus aggregation. Lysozyme was chosen as the model protein as it is well characterised (see Section 11.1).

In Chapter 2 a review of *in vivo* and *in vitro* refolding is given. It is shown that folding within the cell should be considered as a precursor to understanding *in vitro* protein refolding. This is illustrated by the emerging use of molecular chaperones in protein refolding. An outline of the current process steps by which inclusion bodies are renatured is given as well as a

review of recent developments that have been shown to enhance refolding yields.

In Chapter 3 the general materials and methods used throughout the project are described in detail. This includes a description of the controls used and the reproducibility of experiments. The specific methods used in each experiment are described in full in the relevant Chapter.

Chapter 4 is a comprehensive study of the environmental parameters which affect the yield of refolding lysozyme. Although a series of studies of the refolding of lysozyme has been performed previously several parameters have not been studied in full and some conflicting results have been reported. This study details which parameters are most influential on the yield of refolding lysozyme.

In Chapter 5 a competitive model for refolding and aggregation is introduced and rate constants for both reactions are calculated. The results of the model are compared with experimental data and critically assessed. Having shown the accuracy of the model a selectivity term is introduced. This term shows which reaction will predominate at different refolding concentrations. Using the model it is shown that the concentration of refolded protein can be increased whilst maintaining a high yield by adding denatured protein in a step-wise or continual manner. The predicted results are then compared with experimental results.

Chapter 6 involves an economic evaluation of the purchase cost of equipment for an industrial scale refolding process. This is based on the data and model developed in Chapters 4 and 5. The aim of the chapter is to highlight that, although it is important to find new methods of increasing the concentration of refolded protein whilst maintaining a high yield, operating at high yield in the refolding tanks may not offer the most economical process. The effect of

the yield and concentration of refolded protein on cost must be considered over the entire process.

In Chapter 8 the effect of GroEL, an *E.coli* molecular chaperone, on the refolding of lysozyme is investigated. A review of the state of the art knowledge of chaperone assisted refolding is given. It is shown that refolding of lysozyme is possible at high concentrations using equimolar concentrations of molecular chaperone and lysozyme. It is also shown that due to its size GroEL is easily recovered using ultrafiltration membranes and that the effectiveness of GroEL as a refolding enhancer is not affected by the recovery process.

2. Protein Refolding: *In vivo* and *in vitro*

In the this chapter, protein folding both *in vivo* and *in vitro* is described. Folding within the cell should be considered as a precursor to understanding *in vitro* protein refolding. This is illustrated by the emerging use of molecular chaperones in protein refolding. *In vitro* protein refolding methods have been recently reviewed (Hlodan *et al.* (1991), Thatcher and Hitchcock (1994)). An outline of the current process steps by which inclusion bodies are renatured is given as well as a review of recent developments that have been shown to enhance refolding yields.

2.1 Protein Folding *In vivo*

The complexity of the cell has meant that *in vivo* studies of protein folding have always proved to be difficult. *In vitro* studies of refolding do not provide an accurate picture of folding within the cell. However, it is generally believed that folding in the cell is sequential and begins with the interaction between neighbouring amino acids.

Unlike in the case of *in vitro* protein refolding, refolding of proteins *in vivo* occurs sequentially. As the refolding polypeptide emerges from the ribosome secondary structures such as α-helices are formed. These secondary structures once formed are likely to take part in the partial formation of tertiary structure prior to translation being completed (Tsou (1988)). The driving force for protein folding is the free energy difference between the native protein and the unfolded molecule. The free energy value for folding and stabilising the native structure of a single-domain protein is generally of the order of 50 kJ/mol (Privalov (1992)).

Protein folding in the cell is generally faster than *in vitro*. It has been shown that the rate of *in vivo* disulphide bond formation is considerably faster than that capable *in vitro* and that di-sulphide bonds can form prior to translation

being completed (Gilbert (1994)). Studies have also shown that aggregation within the cell is extremely rare unless at elevated temperature or when expressing mutant proteins (Gething *et al.* (1989)). When aggregation does occur the partially folded polypeptide can often be isolated in complexes with specific proteins, notably chaperones and foldases (Bochkareva *et al.*(1988)).

In vitro studies have shown that the rate limiting steps in refolding are often the cis-trans isomerisation of peptidyl-prolyl bonds and di-sulphide bond formation. There is considerable evidence to suggest that the aforementioned foldases and molecular chaperones play an important role in controlling *in vivo* protein refolding (Gething and Sambrook 1992).

Foldases accelerate cis-trans isomerisation (e.g. Peptidyl-Prolyl Isomerase, PPI) and di-sulphide bond formation (e.g. Protein Di-sulphide Isomerase, PDI). Both have been shown to improve refolding yields *in vitro* and are found at high concentration in both eukaryotic and prokaryotic cells.

Molecular chaperones do not accelerate refolding but seem to prevent the formation of aggregates/misfolded protein. Chaperones bind to misfolded proteins, most then, through the hydrolysis of ATP release the protein in a more disorganised state allowing the protein to refold. Chaperones are numerous and diverse. Like foldases they can be found in high concentrations in all cells. As research continues it seems that there is a complex co-operative inter-action between the different types of chaperones. Chaperones are presently considered to be the most exciting area in protein refolding and are discussed further in Chapter 7.

Many proteins are composed of more than one polypeptide chain and the chains associate by non-covalent bonds to form the native quaternary structure. These proteins are classed as multi-subunit or oligomeric proteins and can be homo- or hetero-oligomers. The most common structures are

dimers and tetramers but a large range of other structures including multi-subunit assemblies exist.

The folding of multi-subunit proteins is more complex than the folding of monomeric (single-chain) proteins as it involves both folding and association reactions. The early stages in the folding of oligomeric proteins are similar to those involved in the folding of monomeric proteins. Thus, subunit polypeptide chains will first fold into subdomains or domains and these will then merge to form structured monomers with native-like tertiary structure. The final step is association and further folding to yield the native state. The formation of the quaternary structure involves a sequence of unimolecular folding and bimolecular association steps (Jaenicke (1987)).

Folding steps must precede association in order to form the correct surface areas to allow subunit recognition. They must also succeed association either to stabilise the native quaternary structure or to form the next intermediate with the correct surface areas to allow another association step to occur. The refolding of monomeric proteins is determined by sequential or parallel first-order folding reactions as rate-limiting steps. In the folding of oligomeric proteins second-order association steps may be rate-determining (Jaenicke and Rudolph (1989)).

Association between subunits of a multi-subunit protein is generally highly specific. This specificity is important given the heterogeneous system of the cytoplasm in which proteins form their correct quaternary structure. The folding and assembly of multi-subunit proteins is very efficient *in vivo* with the native quaternary structure formed in seconds or minutes (Seckler and Jaenicke (1992)).

When considering the use of expression systems to produce eukaryotic proteins it is important to remember that the environment in which they are required to fold is quite different from their natural situation. The protein

may naturally be expressed at very much lower levels accompanied by co-expression of chaperones, or other "foldases", and the protein may be subject to post-translational modifications or may be destined for secretion. The environment within the *E coli* cytoplasm may not be the same: it may have proteases present and different chemical properties, *i.e.* redox potential, pH and protein concentration (Thatcher and Hitchcock (1994)). As the protein is engineered to be overproduced in *E coli* the additional metabolic burden is likely to put a strain on the host cell. It is not surprising, that under such conditions inclusion body formation is so prevalent.

In vivo protein folding has been extensively studied by King and co-workers (Mitraki *et al.* (1991), Haase-Pettingell and King (1988), Sturtevant *et al.* (1989)). Their work supports the theory that proteins expressed in *E.coli* place a metabolic burden on the cell. They studied the effect of temperature sensitive mutations (*tsf*) and suppressor mutations on the folding and assembly of the tail spike endorhamnosidase of phage P22. This protein is a highly stable homotrimer and provides a model for inclusion body formation *in vivo*. The expression of the native, wild type protein occurred with about 25% efficiency at 39ºC. The protein expressed at lower temperatures (20-30ºC) was active and had similar thermostability characteristics to the wild-type molecule. It was postulated that a thermolabile intermediate exists, which at high growth temperatures is converted into an aggregate, and at lower temperatures favours the native protein. It was found that the tendency to form aggregates depended on the rate of expression and the rate of refolding. If the rate of expression exceeds the rate of refolding, co-expressed proteins have the ability to come into contact with one other and aggregate prior to reaching their native state.

Much has been learnt about protein folding in the cell over recent years. It now seems that as the primary sequence information is passed from tRNA to the actual peptide structure that the polypeptide begins to refold

immediately. As the polypeptide emerges from the ribosome it associates with other helper proteins which ensure that it reaches its native state. This process is extremely reliable and efficient and only seems to break down when the cell is under stress either due to unfavourable growth conditions or overexpression of foreign proteins. This does not mean that Anfinsen's prediction that all the necessary information for a protein to refold is contained in the primary sequence is incorrect it merely means that *in vivo* there exists auxiliary mechanisms in the cell he had not anticipated.

2.2 *In vitro* Protein Refolding

In vitro refolding has been studied extensively since Anfinsen(1973) first refolded ribonuclease. It has been used as a tool to try to establish the elusive link between the primary sequence of a protein and the final tertiary structure of the native molecule. To date despite a great deal of research this link still evades us. During this time refolding has established itself as an important industrial process. Protein refolding *in vitro* covers an immense amount of work. In the following section I have tried to describe the aspects which are considered relevant to improving the overall refolding process.

2.2.1 Principles of Refolding

In inclusion bodies the recombinant protein is in a misfolded form, and it is likely that the disulphide bonds are incorrectly formed. The first step of renaturation is to isolate the inclusion body using homogenisation to cleave the bacterial cells open and centrifugation to separate the inclusion body from the cell debris. The next step is to solubilise the protein using strong chaotropes such as guanidine hydrochloride or urea. These chemicals solubilise the protein by increasing the solubility of non-polar residues, diminishing the hydrophobic interactions which hold the protein together. The difference between these two denaturants and the implications for refolding have been studied by Matsubara *et al.* (1992). Solubilisation usually

occurs under a reducing environment to break the disulphide bonds. The result of this step is a soluble but misfolded protein. The protein will refold to its correct structure as the denaturant concentration is reduced. This is usually accomplished by diluting the denatured protein solution into a refolding buffer. This buffer should be at the correct pH and redox potential to enable the disulphide bonds to reform by "oxido-shuffling."

Once the denaturant concentration is reduced the protein refolds at rate equivalent to first order kinetics (Kiefhaber *et al.* (1991)). The unfolded protein folds very quickly (2-10 milliseconds) to an intermediate form which has a large amount of secondary structure. There is a slow transition from the intermediate to the native form; this reaction is the rate-limiting step in protein refolding and may take minutes to complete. A simplified reaction scheme is shown in Figure 2.

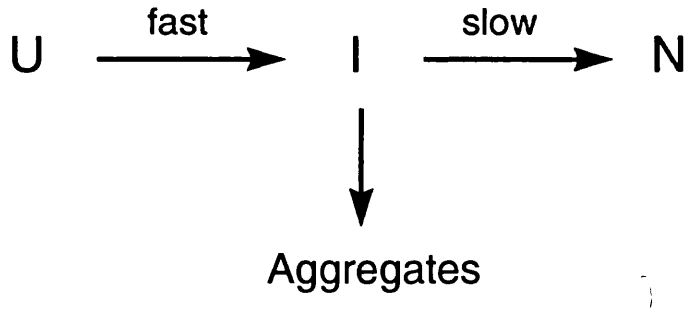

Figure 2-1 Simplified reaction scheme for protein refolding.

U is the unfolded protein, I is the intermediate form which has some secondary structure and N is the native, folded molecule. Aggregates are formed by the interaction of partially folded intermediates.

During refolding non-productive aggregation reactions occur resulting in the formation of stable aggregates which do not re-dissolve upon dilution. Aggregation results from the binding of exposed hydrophobic sites of the folding intermediate (Georgiou and Bowden (1991)). These interactions were thought to be specific as the presence of other proteins did not increase aggregate formation during refolding of tryptophanase (London *et al.* (1991)).

However, Goldberg *et al.* (1991)) found that the refolding of turkey lysozyme was inhibited by the presence of BSA. Aggregation, which involves at least two intermediate molecules interacting has an apparent reaction order of two and is dominant at high protein concentrations. Therefore, in order to reduce aggregation, the protein is diluted to very low concentrations, which may be of the order of 1-10 μg/ml. Much of protein refolding research is focused on trying to minimise aggregation reactions whilst keeping the yield and concentration of the protein folding reaction as high as possible . A schematic flowsheet of the refolding process is shown in Figure 3.

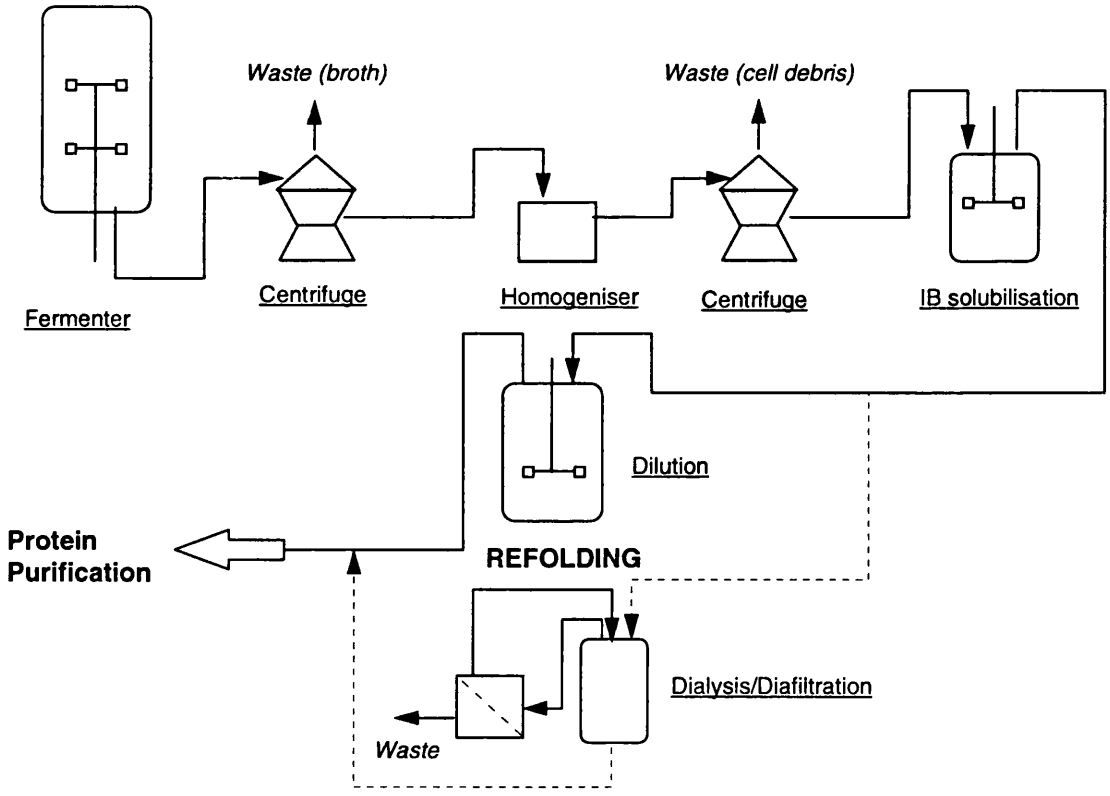

Figure 2-2 Process flowsheet for the renaturation of proteins from inclusion bodies.

2.2.2 Inclusion Bodies

When first discovered inclusion bodies were considered a nuisance, a stumbling block in the production of recombinant proteins. Now they are recognised as a valuable process alternative to the production of soluble protein. They provide a source of easily purified highly enriched protein at an early stage of separation. They are resistant to proteolytic attack and because the protein is inactive they provide an ideal route for the production of protein which is lethal to the host cell.

After fermentation, during which the expressed protein forms inclusion bodies, the first step for the recovery of active protein is isolation of the inclusion bodies. This involves breakage of the cells to release the cell contents including the inclusion bodies. Breakage of the cells can be achieved by high pressure homogenisation or sonication and may be made more effective by pre-treatment with lysozyme, which weakens the cell walls. Prior to breakage the cells may be harvested but the fermentation broth can also be homogenised directly. Initially the cells are suspended in an appropriate buffer and cooled before lysis. Inclusion bodies are highly resistant to shear forces and remain intact after breakage compared to other cellular structures. For the process to be efficient >95% cell breakage is required as unbroken cells will co-purify with the inclusion body fraction resulting in contamination (Thatcher *et al.* (1995)).

The inclusion bodies can be separated from the cell lysate by centrifugation at relatively low velocities. (Taylor *et al.* (1986)). As they are approximately 50-100 % more dense than any other cellular matter they sediment more rapidly than the rest of the cell debris and soluble components. Contaminants such as proteases that could degrade the recombinant protein (Babbitt *et al.* (1990)) can be removed by washing the cell lysate and/or inclusion body pellet with a buffer solution containing detergent without affecting the inclusion body protein (Rudolph (1995), Michaelis *et al.* (1994)). It may also remove *E. coli*

membrane proteins (Georgiou, and Bowden (1991)). In some cases low concentrations of chaotropes have been used to purify the inclusion body during washing. (Hejnaes *et al.* (1992)). Often the viscosity of the lysate is too high at this stage due to unsheared DNA. In this case Dnase treatment should be included prior to inclusion body washing (Rudolph (1990)). This last step can be repeated until a high (>90%) inclusion body content is achieved.

Titchener-Hooker *et al.* (1991) have investigated the engineering parameters involved in the isolation of prochymosin inclusion bodies from *E.coli.*. They found that the performance of each step in the isolation process depended considerably on the preceding steps. By varying fermentation conditions, e.g. antibiotic concentration and initial inoculum volume the size of inclusion body can be increased by 7% and 12 % respectively. This not only increases productivity but also leads to easier separation. With respect to homogenisation they showed that inclusion bodies are extremely resistant to shear and that 3 to 5 passes reduces the mean cell debris size by 10% and a further 10% reduction can be achieved by increasing the passes to 10. The effect of washing inclusion bodies on refolding has not been thoroughly investigated. If purification is necessary it may be advantageous to purify the protein post solubilisation via chromatography where the yields and purification are higher. The exact method used would depend on the cost and is likely to depend on the protein and expression system used.

The inclusion bodies may also be separated by membrane filtration which uses the size difference between inclusion bodies, cell debris and soluble components. Although this technique has been used successfully (Forman *et al.* (1990)) there are also problems associated with it. These including fouling, leading to low productivity and contamination with similar sized cell wall debris (Thatcher and Hitchcock (1994)). In the case of contamination a further extraction step is required to remove the contaminating proteins.

2.2.3 Solubilisation of Inclusion Bodies

Once the inclusion bodies have been isolated the next step is solubilisation. Inclusion bodies are held together by non-covalent forces, mainly hydrophobic interactions, van der Waal's forces hydrogen bonds and electrostatic effects (Georgiou and Bowden (1991)). Solubilisation involves disruption of the non-covalent interactions using chaotropic reagents such as guanidine and urea and the breakage of incorrectly formed di-sulphide bonds using reducing agents such as dithiothreitol. The most commonly used solvents for solubilisation are guanidine hydrochloride and urea and these are used at concentrations of around 4-6M and 6-10M respectively. The concentration of solvent that dissolves the inclusion body is related to the concentration which unfolds the native protein. Guanidine hydrochloride is often preferred to urea as the presence of isocyanate in urea solutions can cause irreversible modifications of amino or thiol groups of the protein.

Occasionally detergents, such as SDS, have been used for the solubilisation but this is only possible when they are used at concentrations above their critical micelle concentration. The major disadvantage is that the detergent may bind to the protein and its removal can be difficult. Detergents may also solubilise any remaining contaminating membrane proteases debris (Thatcher and Hitchcock (1994))

If the expressed protein contains disulphide bonds then in the inclusion body state it is likely that incorrect disulphide bonds have been formed (Shoemaker *ei al.* (1985)). These bonds are subsequently broken by including a reducing agent in the solubilisation solution. The use of low molecular weight thiol reagents such as 2-mercaptoethanol or dithiothreitol in the presence of EDTA, which prevents air oxidation, provides the necessary reducing conditions. The resulting solution containing the solubilised inclusion bodies is then centrifuged to remove any remaining insoluble material.

After solubilisation, further purification of the solubilised inclusion body protein is not usually necessary unless there are impurities, such as proteases, present which will interfere with the refolding process. London *et al.* (1974) have shown that *E.coli.* contaminants have no effect on the refolding of tryptophanase. Weir and Sparks (1987) showed that the refolding of recombinant human interleukin-2. (IL-2) was affected solely by IL-2 concentration and that the concentration of contaminating proteins had no effect on the refolding yield obtained. If further purification is necessary the methods that tend to be used are reversed-phase or hydrophobic interaction and size exclusion chromatography (Rudolph (1980), Chaudhuri (1994)).

2.2.4 Formation of Correct Di-sulphide Bonds

Solubilisation of inclusion bodies of proteins containing di-sulphide bonds often requires the addition of thiol reducing agents to break inter-molecular bonds. Once broken these bonds must be reformed. The number of possible combinations of pairs of thiol groups on a protein increases rapidly with the number of thiol groups present. (See Table 1)

Table 1 The number of possible combinations of di-sulphide bonds as a function of the total number of di-sulphide bonds in the protein.

Number of di-sulphide bonds	Number of possible combinations
1	1
5	945
10	654729075
15	6190283353629375

In the case of RNaseA studied by Anfinsen (1973) there are 4 di-sulphide bonds with a possible 105 different combinations. Tissue plasminogen activator has 17 di-sulphide bonds which yields a staggering 2.2×10^{20} possible combinations. This a merely a statistical representation and takes

no account of the steric and energetic restraints on the number of combinations possible. Creighton studied bovine pancreatic trypsin inhibitor, a small protein of 58 residues containing 3 di-sulphide bridges. This group found that during refolding only 4 different di-sulphide bonded species out of the 15 possible conformations occur. However no correlation was found between reaction time and conditions and the prevalence of any particular species. This suggests that although the number of possible combinations of disulphide bonds is limited. (presumably due to the steric restrictions imposed by the collapse of the unfolded molecule) that di-sulphide does occur.

To reform di-sulphide bonds after solubilisation of inclusion bodies the reduced thiol groups must be oxidised. This was first achieved by bubbling air through the a solution of the reduced protein. (Sela *et al.* (1957)). This simple procedure produced a yield of 20% active refolded protein. Other studies into the air oxidation of reduced di-sulphide bond proteins were performed at the time. However, yields were typically low and results were not easily reproduced. This was due to experimental procedures being difficult to reproduce accurately. Different bubbling rates gave rise to different quantities of surface denaturation and the procedure was extremely sensitive to trace levels of certain metal ions.

The poor yields and low reliability lead to the discovery of mixed thiol systems. This involved oxidative regeneration of di-sulphide bonds by the addition of low molecular weight thiols in the reduced and oxidised form to the refolding buffer. Reduced and oxidised glutathione are generally used. Usually ten times more thiol form (GSH) than disulphide form (GSSG) is used. The thiol group is used at 10-50mM. Although not identical this is similar to conditions found *in vivo*. The thiol and di-sulphides accelerate the formation of correct and breaking of incorrect di-sulphide bonds in the refolding protein. Thiols are only reactive in their ionised thiolate form. Therefore for the refolding of proteins containing di-sulphide bonds the pH of

the refolding buffer should be neutral or basic. A more detailed description of di-sulphide chemistry is given by Gilbert (1994).

Di-sulphide bond formation *in vivo* is catalysed by the prescence of protein di-sulphide isomerase (PDI). The effect of this enzyme has been investigated for a number of proteins. PDI catalyses the rate of di-sulphide exchange in the refolding protein. Freedman has extensively studied the effect of PDI and has written a comprehensive review on the subject (Freedman (1989)). This leads to a more rapid formation of stable refolding intermediates and consequently higher refolding yields.

2.2.5 Refolding Proteins

The last step for the recovery of native, active protein involves refolding and reactivation of the denatured protein. This is achieved by removing the denaturant and reducing agent and providing the correct environment for refolding of the protein. The options available for refolding include dilution, dialysis and diafiltration.

2.2.5.1 Dilution

Dilution of the denatured protein into a refolding solution is the simplest procedure and has been used frequently to refold proteins (Thatcher and Hitchcock (1994)). The denaturant and, if present, the reducing agent concentration are reduced in one step to a level which allows refolding to take place. The protein concentration is also lowered in this diluting step helping to prevent aggregation and maximise the yield of refolded protein. This option has the disadvantage that in keeping the protein concentration low it involves large process volumes (Rudolph (1991)). Moreover the sudden change in denaturant concentration can occasionally lead to aggregation as some proteins are less soluble in the unfolded and partially folded state than in the native state. (Thatcher and Hitchcock (1994)).

Formation of the correct disulphide bonds during refolding is achieved by maintaining a redox potential with low molecular weight reduced and oxidised thiol compounds. The combination of reduced and oxidised glutathione is often used at molar ratios between 10:1 to 5:1. The thiol-disulphide exchange system increases the rate and yield of protein renaturation/reoxidation by reshuffling any incorrect disulphide bonds formed.

Refolding of some proteins can be improved by using a two-step dilution. The protein solution containing a high denaturant concentration is initially diluted to an intermediate denaturant concentration or the strong denaturant is replaced by a weaker one before undergoing a second dilution to remove the denaturant. The first dilution allows the protein to fold to a stable state in the presence of a low concentration of denaturant. This prevents aggregation by shifting the equilibrium away from aggregating folding intermediates towards intermediates which contain a high degree of native structure but which do not aggregate. The second dilution removes the denaturant and the protein can fold to its native, active structure. Although this procedure has been successfully applied to chymotrypsinogen (Orsini and Goldberg (1978)) and carbonic anhydrase B (Cleland and Wang (1990)) it has also been shown to have no effect on others. Weir and Sparks (1987) found that refolding interleukin-2 into sub-denaturing conditions; 2M GuHCl, had no effect on the recovery of the active protein. If a concentration of denaturant can be found which exposes these areas enough to allow the refolding of the protein to proceed but remains high enough to prevent intermolecular reactions then two-stage folding will be successful. The success of this method is likely to depend on the equilibrium between the denatured state, the intermediate state and native state for different denaturant and protein concentrations.

2.2.5.2 Dialysis

Dialysis is the transfer of solute molecules across a membrane by diffusion from a concentrated solution to a dilute solution. A simultaneous diffusion of solvent molecules (in this case water) occurs in the opposite direction. The membranes that are used can be porous or non porous. In refolding porous membranes are used. Separation in dialysis occurs to differences in the size and diffusion rates of the different solutes.

Dialysis can be used in protein refolding to reduce the denaturant concentration and at the same time supply the refolding solution to the denatured protein. The dialysis membrane allows passage of small molecules through from both directions while retaining the large protein molecules inside. As the denaturant concentration decreases and is replaced by refolding solution the conditions become favourable for refolding of the protein. Dialysis allows the denaturant concentration to be reduced gradually rather than by the sudden change that occurs with dilution (Thatcher and Hitchcock (1994)). This may be beneficial for some proteins reducing the possibility of aggregation and increasing the yield of native protein by controlling the equilibrium of folding intermediates in a manner which reduces the concentration of aggregating species (Vicik and De Bernadez-Clark (1991). Dialysis will not give high refolding yields if folding intermediates are prone to aggregation. Dialysis has been used to refold tryptophanase (London *et al.* (1974)) and bovine growth hormone (Builder and Ogez (1986)).

The disadvantage of this process is that it is slow, as it is mass transfer controlled (Chaudhuri (1994)). Due to this fact it is likely to be uneconomical when used on an industrial scale.

2.2.5.3 Diafiltration

Diafiltration separates a solute from a solution by forcing the solvent to flow through a membrane by applying a pressure across the membrane. The rate

of solvent flow, flux is a function of the membrane properties solvent conditions and is directly proportional to the pressure applied. In diafiltration the membrane is porous and the size of the pores determines which solutes pass through. Molecules larger than the pores are retained. The process can be run at constant volume or in concentration mode. This allows control of the concentration of the solutes.

Prorennin (Builder and Ogez (1986)) and carbonic anhydrase (Vicik and De Bernadez-Clark, (1991)) have been refolded using diafiltration. A membrane is chosen for the denatured protein with the optimum molecular weight cut-off which will allow the denaturant molecules to pass through and leave the protein molecules behind. The refolding solution is added to the refolding tank and at the same time the denaturant is removed. Diafiltration allows a degree of control over parameters such as pH, ionic strength and protein and denaturant concentrations.

An advantage of the technique is that the process time is much shorter than dialysis and this factor is important when carrying out refolding on a large scale. A disadvantage of the system is the unproductive interaction between proteins and membranes. Denatured proteins have been shown to pass through membranes with molecular weight cut-offs lower than the molecular weight of the protein (West,S. unpublished results). Although many advances have been made in reducing protein binding to membranes this phenomenon is likely to pose a significant problem especially as the protein is in the denatured/intermediate state when hydrophobic residues are exposed. If high fluxes are required concentration of solutes near the membrane surface may be significant, if the concentration of refolding protein at the membrane surface rises too high than aggregation will predominate. (See Figure 2-3)

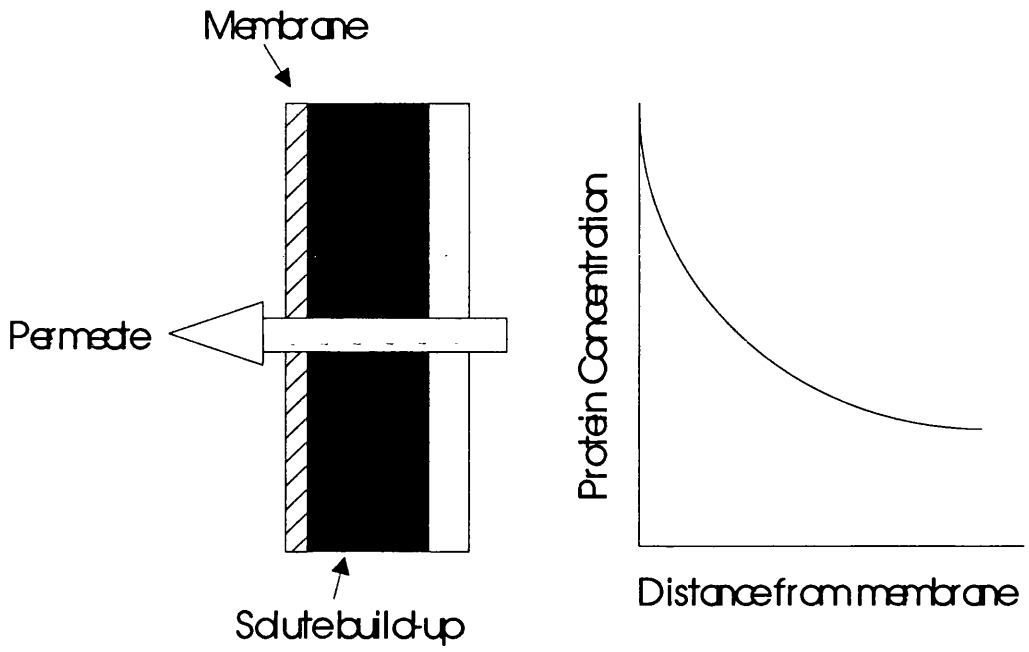

Figure 2-3 Solute build up in membrane processes

In summary, certain proteins have intermediate folding structures which aggregate. These structures proliferate at intermediate denaturant concentrations. Diafiltration and dialysis expose the refolding protein to intermediate denaturant concentrations for long periods of time. For proteins with folding intermediates which are susceptible to aggregation dilution will be the most effective method of refolding. Some refolding intermediates are stabilised by the prescence of denaturants. In this case dialysis and diafiltration may provide a superior alternative. As of yet no correlation has been shown between the properties of recombinant proteins and which refolding procedure is best. As such the optimum procedure has to be determined on a case by case basis.

2.2.6 The effect of environmental parameters on refolding.

Temperature can have a significant effect on protein folding. Although this has not been widely studied. The majority of refolding experiments are performed in the range 0-40°C with room temperature (20-25°C) most

commonly used. Often in this range an increase in temperature results in an increase in the rate and yield of renaturation but at temperatures well above this range the efficiency of folding is decreased (Fischer *et al.* (1993), Jaenicke and Rudolph (1989)). However, the studies of several proteins including rhodanese (Mendoza *et al.* (1991)), the dimeric form of Rubisco (Gatenby, *et al.* (1990), Viitanen *et al.* (1990)), Fab fragments (Buchner and Rudolph (1991)) and the phage P22 tailspike protein (King *et al.* 1988. 1989, 1991) have shown that at high temperatures (30-40°C) little reactivation occurs whereas at low temperatures reactivation increases with a maximum around 10°C.

The solvent conditions during refolding are extremely important. It is necessary for the denaturant concentration to be reduced to a level which allows the protein to refold. The residual denaturant concentration governs which intermediate species proliferate. As the concentration of a denaturant increases, the solubility of both apolar groups and alkanes increases. Considering this information alone one would assume that as denaturant concentration increases the solubility of a given protein will increase. It is important however to remember that due to the equilibrium existing between the native and the denatured states that an increase in denaturant concentration also increases the number of denatured molecules in the system. These molecules may then form aggregates which are insoluble. An optimal denaturant concentration would be one which is low enough to allow the protein to refold to a molten globule type structure which is fairly compact but which would also inhibit aggregation by keeping the protein soluble. The concentration of denaturant at which aggregating species predominate is different for different proteins and denaturants and as such no guidelines exist for a general optimum.

In all literature reviewed increasing either the initial or final concentration of protein in a refolding system decreases the obtained yield. (Buchner and Rudolph (1991), Goldberg *et al.* (1991), Rudolph *et al.* (1995)). The decrease in

yield is due to unfavourable intermolecular reactions which lead to aggregation. As the concentration of refolding protein these interactions become prevalent and the observed yield of refolded protein is reduced. As such protein concentrations are kept low during folding to prevent aggregation. Typically, to achieve high yields concentrations of less than 0.01 mg/ml are used. In a commercial environment this requires the use of large volumes and often yield is sacrificed in favour of a higher final concentration of renatured protein (Forman *et al.* (1990)).

Rudolph suggested that one method to increase the protein concentration during folding would be to add the unfolded protein to the refolding solution in a stepwise manner (Rudolph (1991)). The protein can be added either continuously or discontinuously (pulse renaturation). The process involves adding a small amount of unfolded protein to the refolding solution and allowing it to fold before adding more unfolded protein. The amount of unfolded protein added at each step must not exceed the concentration where aggregation becomes dominant. This method relies on native refolded protein not interacting with newly added refolding protein. This system has not been investigated in detail but in theory in this system the protein concentration can be increased to a higher level than can be achieved by a single dilution of the unfolded protein whilst maintaining a high yield.

2.2.7 Modelling Refolding

Like *in vitro* refolding experiments, modelling of protein refolding has been aimed at trying to establish a link between the primary sequence of the polypeptide chain and the tertiary structure of the native molecule. Several models have been formulated. (Karplus and Weaver (1976), Kim and Baldwin (1982), King (1989)). These models include biased random search, nucleation growth, diffusion-adhesion-collision, and sequential folding. Generally these models conform to the experimental hypotheses about in vitro refolding and

can be described by the rapid formation of a hydrophobic compact structure followed by the slow shuffling of the protein to its native state.

Karplus and Weaver first suggested the diffusion-collision model. This suggests that upon removal of the denaturant micro-domains are formed and then collide to form a stable structure. This model has been supported by NMR studies on refolding. (Wright *et al.* (1988)) and by other in vitro refolding experiments which suggests that a stable refolding intermediate is formed prior to a final rearrangement of sub-domains which leads to the formation of the native protein (Kuwaijima (1989), Ptitsyn *et al.* (1990)). The main driving force is the interaction between hydrophobic residues and the solvent.

The above models are aimed at understanding the biophysical aspects of refolding and do not take into account aggregation. A simple model for refolding and aggregation was suggested by Kiefhaber *et al.* (1991). They suggested that refolding and aggregation of lactic dehydrogenase could be approximated to a first order refolding reaction competing against a second order aggregation reaction. They calculated the rate constants for both reactions from experimental data in a paper by Zettlemeissel *et al.* (1979). The model showed good agreement with the experimental data for refolding and accurately predicted the expected yield from a given the concentration of lactic dehydrogenase in the refolding buffer. The model however is only valid for the conditions under which the rate constants for refolding and aggregation were calculated. The overall yield of refolding is strongly dependant on the solvent conditions of the renaturation buffer. These were not taken into account in the model.

2.2.8 Refolding Enhancement

As refolding has grown in industrial importance more research has been aimed at trying to increase the concentration at which proteins can be refolded whilst maintaining a high yield. Refolding yields have been

enhanced through the addition of various chemical species. These include sugars, amino acids, surfactants, polymers and other proteins.

The use of the amino acid L-arginine added to the refolding buffer has been found to increase the renaturation yield of antibody fragments (Buchner and Rudolph (1991)) and tissue plasminogen activator (Rudolph *et al.* (1991)). Arginine added at a concentration of 0.35 - 0.50 M resulted in a 60% increase in active Fab fragments. At a concentration of 0.5 M increased the yield of tissue plasminogen activator (tPA) from 5% to 95%. This is an extremely large enhancement but the authors do not quote the concentration of protein being used and as such the significance of the result is very difficult to assess. The authors believe that the chaotropic nature of L-arginine destabilises incorrectly folded and incorrectly disulphide-bonded structures allowing the molecules to proceed along the correct folding pathway (Rudolph *et al.* (1991)). If the binding of L-arginine to the misfolded structure is found not to be a protein-specific interaction this method may find widespread applicability. However, if the explanation for the enhancement given by the authors is correct then it is likely that the applicability of this method will be the same as two-stage dilution and it will be strongly dependant on the stability of the refolding intermediates of each particular protein. This method will probably be protein specific and depend on the equilibrium between the intermediate state and refolded state caused by the concentration of arginine in the system.

Polyethylene glycol (PEG) has been used to enhance refolding yields (Cleland and Wang (1990)). The use of PEG concentrations of 3 g/l in the refolding buffer increased the refolding rate of carbonic anhydrase B threefold. The protein concentration was 0.5 mg/ml. The observed increase in yield has been attributed to either PEG transiently bonding with the refolding intermediate preventing it from aggregating or alternatively the observed increase in the yield may be a result of PEG reducing the diffusion of carbonic anhydrase and effectively causing the aggregation reaction to become mass transfer limited.

The actual explanation is likely to be a combination of the two theories. This method has also been applied to refolding rDNAse from *E coli* inclusion bodies, and tissue plasminogen activator and γ-interferon derived from CHO cells. PEG refolding is not likely to find application on an industrial scale. For it to be effective it is required at concentrations significantly higher than the concentration of the product. The expensive of the additive would preclude it from any refolding process.

In a very novel approach, monoclonal antibodies have been used to refold the S-protein fragment of ribonuclease A (Carlson and Yarmush (1992)). The authors suggest that the use of a specific antibody to the protein acts as a template for the protein to refold upon. The S-protein was reduced and then mixed with a monoclonal antibody raised to the native S-protein. The correct redox potential for refolding was provided using glutathione. Refolding was stopped after about 24 hours by carboxymethylation of remaining reduced sulphydryl groups. The S-protein was recovered from the monoclonal antibody and any misfolded protein by gel filtration. In the absence of the monoclonal antibody the recovery of enzymatic activity was 13%, whereas it rose to 54% in the presence of the antibody. Although this approach was successful, the requirement for monoclonal antibodies would make this an expensive refolding process to be used for manufacturing. However, if the theory that refolding can be enhanced by the prescence of a template then it would be interesting to study the effect of the protein substrate/target or an analogue of these on the effect of refolding.

Hagen *et al.* (1990) have used surfactants to encapsulate unfolded protein. Each micelle contains one protein molecule preventing aggregating species to come into contact with each other. Although they achieved some degree of success they encountered problems with the surfactants binding irreversibly to proteins with strong hydrophobic moieties.

In a similar study Zardeneta and Horowitz (1987) refolded rhodanese at 0.2mg/ml with greater than 45% yields by using mixtures of Triton-X 100/lauryl maltoside and phospholipids. The micelle contains both polar and non-polar moieties in its head groups which can bind to the exposed sites of the refolding protein unlike in the system of Hagen *et al.* (1990) where only polar head groups were present. At 0.02 mg/ml they accomplished 94% refolding, higher than any other method for rhodanese including chaperone (Martin *et al.*, 1991) and detergent (Tandon and Horowitz, 1987) systems.

PPI and PDI have been used *in vitro* to enhance refolding yield. Cis-trans proline isomerisation and disulphide interchange are recognised as being the major kinetic barriers in refolding. A complete review of the role of proline isomerases in refolding has been written by Schmidt *et al.*, (1993). Freedman (1989) has written a comprehensive review of the role of protein disulphide isomerases. Both methods improve refolding yields. However, isomerases are expensive to produce and although no economic study has been completed, it would be difficult to justify using isomerases when other cheaper methods of obtaining high concentrations of refolded protein at high yield are just as efficient.

In *in vitro* refolding it has been shown that refolding is determined by the amino acid sequence alone. It has been shown that refolding in the cell involves other proteins. The predominant role of these proteins, known as molecular chaperones, appears to be preventing the incorrect intermolecular association of unfolded polypeptide chains which results in aggregation. An interesting review on this subject has been written by Hartl *et al.* (1994) and more recently Hartl has written another review in which he discusses the latest developments in the understanding of molecular chaperone interactions (Hartl 1996).

As with the isomerases chaperones are expensive, they often require ATP to be present and it has been suggested that they are unlikely to find an

industrial application in *in vitro* refolding. It has been suggested that if chaperones could be expressed at the same time as the desired protein it may increase dramatically the amount of soluble protein that can be produced per cell.

Cleland (1993) gives some guidelines for the characteristics of the ideal folding enhancer. The species should be cost effective, it should inhibit protein aggregation without interfering with folding, and it should be easily removable from the native protein after folding. The issue of cost is very important when considering refolding on a significant scale. The mass ratio of folding enhancer to protein may be used as an indicator of the cost effectiveness of the reagent. In many cases the cost of the enhancer may outweigh the benefits. In all cases above the enhancer has been added to the refolding medium.

In addition to enhancing refolding yields by adding enhancers to the refolding medium several physical methods of improving refolding have been tried.

Werner *et al.* (1994) refolded RNaseA using gel filtration chromatography. Gel filtration inhibits aggregation by preventing folding intermediates coming into contact. It also has the advantage that refolding and purification are performed in the same step. Similar plug-flow type reactors have been investigated by Terashima *et al.* (1996) and Hamaker *et al.* (1996). This mechanism has been exploited for the refolding of lysozyme. Batas and Chaudhuri (1996) successfully refolded lysozyme loaded onto a column at 80 mg/ml. The final concentration of refolded lysozyme was 0.71 mg/ml and of the 120 mg of denatured protein applied to the column 55 mg were recovered. This corresponds to a yield of 46%. To achieve this yield using dilution the protein would need to be refolded at approximately 0.1 mg/ml. This would correspond to a volume of 1.2 dm^3 of refolding buffer which then needs to be concentrated seven fold before it is in a similar state to the product of the gel column. The advantages of the process are obvious. However, the scale-up of

gel chromatography is not easy and is often associated with loss of efficiency. Capital costs are well in excess of those of a simple dilution tank despite the larger volumes involved. Refolding small molecules may be efficient in a gel column but the refolding of larger proteins like tissue plasminogen activator which take several hours to refold would lead to long residence times needed in the column which would increase the size of the column significantly. This method of refolding is extremely interesting and may well prove useful for the small scale production of relatively small proteins but it is unlikely to find use on an industrial scale.

2.2.9 Protein Aggregation

The aggregation/precipitation of native protein molecules has been studied in depth. The effect of temperature, pH, type of salt and salt concentration have all been investigated for a number of proteins (Bell *et al.* (!983)). Ironically, these studies have been aimed at causing the native molecule to aggregate so as to effect separation of proteins. The aggregation of refolding proteins has not been studied in depth and little is still known about the exact intermolecular interactions which lead to the formation of the precipitate.

From early studies in protein refolding aggregation of protein molecules has been known to be the cause of low refolding yields (Anfinsen (1973)). The first work specifically aimed at aggregation during refolding was performed by Zettlemeissel *et al.* (1979). These studies revealed that aggregation competed with refolding. Like refolding the rate and extent of reaction was found to depend on the protein concentration (Zettlemeissel *et al.* (1979), Rudolph *et al.* (1979), Cleland and Wang (1990)).

Seckler *et al.* (1989) studied the difference between *in vivo* and *in vitro* aggregation for the refolding of the Phage 22 tailspike protein. They showed that aggregation was more prevalent *in vitro* and postulated that the

difference in the observed kinetics of refolding was due to the lack of chaperones and foldases in the refolding buffer.

Aggregation has been shown to be dependent on the denaturant concentration in the refolding buffer. As previously mentioned in the section on the effect of solvent conditions on refolding, this is due to the fact that the solvent conditions dictate which folding intermediates dominate. Mitraki *et al.* (1987) showed that there exists a critical concentration of denaturant at which a protein is most likely to aggregate. This has been supported by a comprehensive study of the refolding pathway of carbonic anhydrase B (CAB) which showed that hydrophobic intermediates formed most rapidly at low denaturant concentration (Cleland and Wang (1990)). These intermediates were identified as being the most likely to form multimers and then aggregates.

Aggregation of refolding proteins is generally considered irreversible. That is strong denaturants are usually required to dissolve them. Transient reversible associations however, have been shown to occur. Brems *et al.* (1987) showed that the rate limiting step in the refolding of bovine growth hormone (bGH) was the dissociation of a transient dimer. A similar association was observed for the refolding of CAB (Cleland and Wang (1990)). Both these groups showed that these transient dimers could be stabilised under specific solvent conditions (CAB 2M GuHCl and bGH 3.7M GuHCl or 8.5 M urea) and that upon increasing the protein concentration these dimers aggregated irreversibly.

Non-covalent forces are the most likely cause of aggregation (Mitraki and King (1989)). However Shoemaker *et al.* (1985) suggested that inter molecular di-sulphide bond formation may be important. Hydrophobic forces are the strongest non-covalent interactions are the strongest and have been suggested to be the driving force for refolding (Dill (1990)). It is thought that they are also responsible for aggregation. Marston (1986) showed that

removing a hydrophobic region from a glycoprotein increased its solubility. Tandon and Horowitz (1986) showed that rhodanese aggregates upon exposure of hydrophobic areas of the protein using GuHCl.

Light scattering techniques have been used to study the kinetics of aggregation. These include the study of lactic dehydrogenase (Kiefhaber *et al.* (1991)). It has been proposed that aggregation can be approximated to a second order reaction. This would be true for the aggregation of monomers where convective forces are not important. This is obviously not the case in the aggregation of refolding proteins where large multimers are formed quickly. However, as a first approximation, the model fits well to the experimental data and is valid under the refolding conditions used.

Aggregation occurs during refolding. This is thought to occur due to the interaction of hydrophobic folding intermediates. The extent and rate of reaction depend strongly on the concentration of protein and the solvent conditions. The reaction can be approximated to a second order reaction and with a rate constant considerably higher than that for refolding.

2.3 Conclusions

It is generally believed that the production of recombinant proteins via inclusion bodies is likely to remain an important manufacturing route for some time to come. Increases in protein refolding yields will be an important objective in process development. It is thought that improvements in protein folding yields and the ability to refold at higher protein concentrations are likely to come from improvements in the refolding step. It is unlikely that new methods for inclusion body isolation and solubilisation will yield step changes in efficiency.

It seems that from an industrial point of view that dilution will remain the optimum method for refolding. Dialysis is too slow a process and despite the advantages of using diafiltration for the refolding of certain proteins, membranes remain expensive. A great deal of work on the solvent conditions required for optimum refolding has been completed and it would seem that they are highly protein dependent. The literature available on the effect of environmental parameters on the refolding of lysozyme is incomplete and in places contradictory. The effect of temperature and pH have been studied previously but only over limited ranges. The effect of the ratio of reducing and oxidising thiol groups in the refolding buffer has been studied and optimised. The effect of adding denatured protein containing reducing agent and denaturant on the refolding yield however has not. The effect of protein concentration on the refolding of lysozyme has been studied by a number of groups. All found that increasing the concentration of protein reduced the yield of native lysozyme but the reported values of the final yield differ significantly. The importance of the effect of the lysozyme concentration in the reduced form prior to refolding on the yield of refolded protein has generally been overlooked.

Many methods of enhancing refolding yield have been studied. These include the addition of a variety of chemicals to the refolding buffer. With the

exception of peptidyl-prolile and di-sulphide isomerases these additives enhance refolding yields by reducing non productive intra and inter molecular reactions. Thereby reducing aggregation and increasing the yield of refolded protein. Several chromatographic methods of refolding have been attempted. This involves preventing individual refolding polypeptides interacting by physical exclusion. All these methods have enhanced refolding yields over straight dilution and yet an industrially attractive possibility has yet to be found. Methods of improving the refolding yield by adopting different process techniques have been sparsely investigated. The only literature available on this method of improving refolding yield is a patent which states that refolding can be enhanced by adding denatured protein to refolding buffer in a step-wise or continuous manner. No reaction engineering justification is given for the observed results. This area of refolding yield enhancement requires further investigation. If proteins can be refolded at higher concentrations without the additional expense of additives or complicated equipment it could drastically reduce process costs. Thus the objectives of this study are to :

- identify the key environmental factors which affect the refolding and aggregation of lysozyme
- model the refolding and aggregation of lysozyme using fundamental reaction engineering principles
- suggest process strategies to enhance the yield and concentration of refolded lysozyme

3. Materials and Methods

Described here are the general materials and methods used throughout Chapters 5, 6 and 8. Any other specific materials or methods used are described in the appropriate chapter.

3.1 Materials

Hen egg white lysozyme (EC 3.2.1.17; 52,000 units/mg), *Micrococcus lysodeikticus* dried cells, oxidised and reduced glutathione (GSSG and GSH), guanadine hydrogen chloride (GuHCl), dithiothreitol (DTT), Tris HCl, potassium di-hydrogen orthophosphate, EDTA and nitric acid were obtained from Sigma Chemicals. (Poole, Dorset). All chemicals were of analytical grade.

3.2 Experimental Methods

Lysozyme was renatured by diluting a sample of denatured reduced lysozyme into refolding buffer under controlled conditions. The recovery of activity over time was monitored by removing a small sample of the refolding protein and performing an activity assay. This involved adding the sample to a solution of dried bacterial cells. Lysozyme breaks down the cell wall of the bacteria. This results in a decrease in the turbidity of the solution. The rate of change of this decrease can then be compared to that of the native protein and the percentage recovery of activity (yield) over time can be calculated.

3.2.1 Preparation of Denatured Lysozyme.

The methods used for the denaturation of lysozyme were adapted from those used by Goldberg *et al.* (1991). For purposes of comparison, three methods of preparation were used. The first two methods differ in the type of denaturant used (i.e. GuHCl and urea) and in the third dialysis to remove the denaturant and the reducing agent.

1. Approximately 2ml of a concentrated solution of lysozyme (approximately accurately 100 mg/m) in 0.1 M Tris HCl pH 8.2 was added to 6ml of a solution containing 5.725 g of GuHCl 0.231 g of DTT dissolved in 0.1M Tris HCl pH 8.2 this solution was then made up to 10ml with in 0.1M Tris HCl pH 8.2 to give a final concentration of 20 mg/ml lysozyme in 6M GuHCl, 0.15 M DTT and 0.1M Tris HCl pH 8.2 which was then left to incubate at room temperature for two hours before use. Denatured protein was prepared as required and stored at 4 °C.

2. Approximately 2ml of a concentrated solution of lysozyme (approximately 100 mg/ml) in 0.1 M Tris HCl pH 8.2 was added to 6ml of a solution containing 6.0g of urea, 0.231 g of DTT dissolved in 0.1M Tris HCl pH 8.2. This solution was then made up to 10ml with in 0.1M Tris HCl pH 8.2 to give a final concentration of 20 mg/ml lysozyme in 10 M Urea, 0.15 M DTT and 0.1M Tris HCl pH 8.2 which was then left to incubate at room temperature for two hours before use. Denatured protein was prepared as required and stored at 4 °C.

3. Approximately 2ml of a concentrated solution of lysozyme (approximately 100 mg/ml) in 0.1 M Tris HCl pH 8.2 was added to 6ml of a solution containing 5.725 g of GuHCl 0.231 g of DTT dissolved in 0.1M Tris HCl pH 8.2. This solution was then made up to 10ml with in 0.1M Tris HCl pH 8.2 to give a final concentration of 20 mg/ml lysozyme in 6M GuHCl, 0.15 M DTT and 0.1M Tris HCl pH 8.2 which was then left to incubate at room temperature for two hours before use. This solution was then dialysed against 1.5 dm^3 of 0.1 M acetic acid for 1 hour, the buffer was then exchanged with another 1.5 dm^3 of 0.1 M acetic acid and left to equilibrate overnight. After dialysis the protein was centrifuged at 10000 rpm in a micro-centrifuge to remove any aggregated material and was then stored at -20 °C or 4 °C if it was to be used immediately.

The Ellman assay was used to verify the complete reduction of the four di-sulphide bonds that lysozyme contains (see Section 3.3.3).

3.2.2 Renaturation of Lysozyme

3.2.2.1 Batch renaturation

The study into batch refolding involved investigating the system and environmental parameters which have an effect on the refolding process. Described here is the general procedure used for refolding In order to avoid unnecessary repetition, how each of the specific variables studied were investigated is explained in detail in each results section.

General method used for the refolding of lysozyme

Renaturation was initiated by adding 100 µl of a 20 mg/ml solution of denatured reduced lysozyme to 200 ml of renaturation buffer to give a final concentration of 0.01mg/ml of lysozyme (0.1M Tris-HCl, pH8.2, 1 mM EDTA, 3mM reduced glutathione and 0.3 mM oxidised glutathione). The renaturation solution was incubated in a water bath at the desired temperature and lysozyme activity was assayed at several time points.

Table 2 The variables and range of variables investigated

Variable	Range studied
Type of denaturant	GuHCl, Urea or Acetic Acid
Protein concentration	0.01 - 0.2 mg/ml
Initial Protein Concentration/ Dilution factor	100 - 1000
pH	pH 5 - pH 8.2
Residual GuHCl concentration	0.01M -0.1 M
Residual DTT concentration	0 075 mM - 0.25 mM
Temperature	4 - 60 °C

3.2.2.2 Fed batch renaturation

Fed batch refolding is essentially cycles of batch refolding performed in the same refolding buffer. This results in a gradual increase in the protein concentration in the refolding buffer.

General method used for fed-batch refolding of lysozyme

Renaturation was initiated by adding 100 µl of a 20 mg/ml solution of denatured reduced lysozyme to 200 ml of renaturation buffer to give a solution of 0.01mg/ml of lysozyme (0.1M Tris-HCl, pH8.2, 1 mM EDTA, 3mM reduced glutathione and 0.3 mM oxidised glutathione) and the subsequent rise in specific activity of the refolding solution was monitored using the activity assay. After a given time another 100 µl of a 20 mg/ml solution of denatured reduced lysozyme was added to the refolding buffer. This process was then repeated 6-10 times depending on the specific experiment. Denatured reduced lysozyme prepared by Methods 1 and 3 were used for the experiment and the results compared. Both the amount of protein added in each addition and the time allowed between consecutive additions were varied. Aggregation was followed at 450 nm. All experiments were performed at 25°C

3.2.2.3 Continuously fed batch renaturation

A 0.1 mg/ml solution of denatured reduced lysozyme was added to 200 ml of refolding buffer (0.1M Tris-HCl, pH8.2, 1 mM EDTA, 3mM reduced glutathione and 0.3 mM oxidised glutathione) using a Watson-Marlow 1010 micro-peristaltic pump (Watson-Marlow Ltd. Falmouth, Cornwall). The flow-rate of the pump was calibrated (See Appendix). As the flow-rates used were so low the feed pipe from the pump was kept immersed in the refolding buffer at all times to avoid droplet formation, which would have simulated fed-batch refolding.

To achieve different rates of addition the concentration of denatured reduced lysozyme added to the system (0.1 mg/ml) was kept constant and the rate of addition was varied. Refolding was monitored using the lysozyme activity assay. Aggregation was monitored at 450 nm using a Cecil Instruments Series 3000 spectrophotometer (Cecil Instruments, Milton Technical Centre, Cambridge). The system was gently stirred using a magnetic stirrer and all experiments were performed at 25°C

3.2.3 Kinetic Study of Aggregation

The kinetics of aggregation of reduced denatured lysozyme were measured in a Hi-Tech Scientific SF-60 series stop-flow spectrophotometer (Hi-Tech Scientific, Salisbury, England). A stop-flow apparatus was necessary due to speed of the aggregation process. A typical stop-flow apparatus is shown in Figure 3-1. The syringes were filled with denatured reduced lysozyme and refolding buffer. Equal volumes of the two solutions were then rapidly mixed and the extent of aggregation was measured.. The dead time was less than 1 ms. The dead time of the equipment is the time it takes the mixture to reach the observation cell. The system temperature was kept at 25 °C using a water bath.

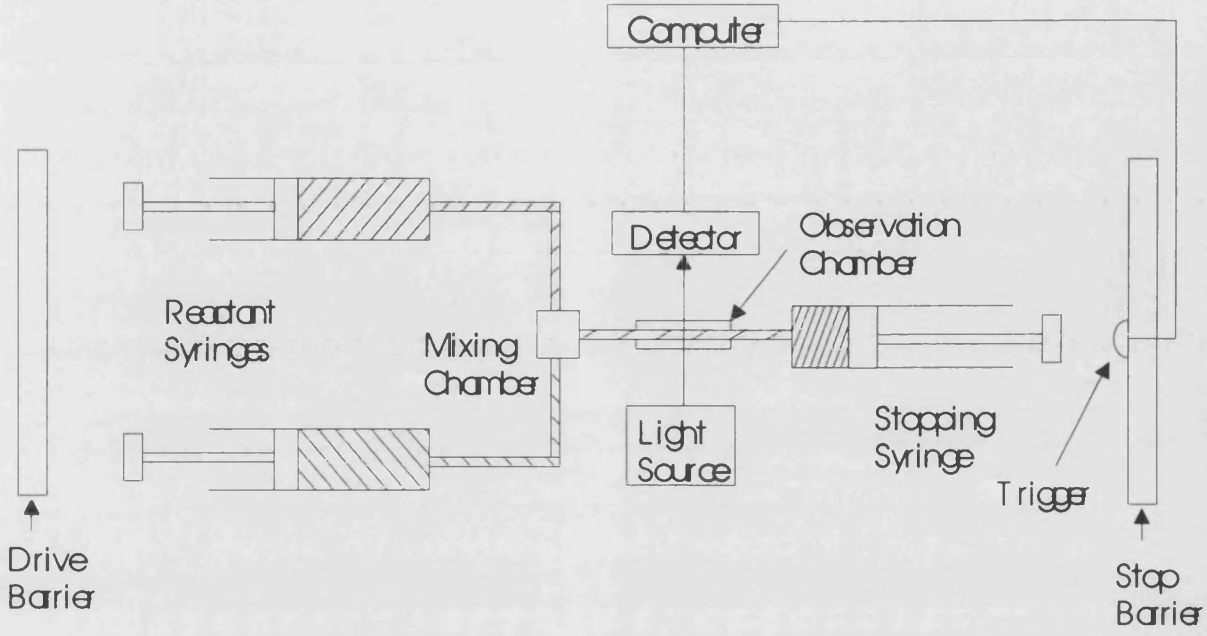

Figure 3-1 A typical stop-flow apparatus

The reaction was followed at 96 wavelengths between 280 and 600 nm. Data was analysed at 450 nm, outside the absorbance spectra of lysozyme. The flow cell was washed with de-ionised water, then with concentrated nitric acid to remove any organic material, rinsed with distilled water, cleaned with ethanol to remove any insoluble organic compounds and then finally flushed with distilled water in-between each run. Unfortunately, the dilution capabilities of the machine were not suitable for dilutions over 1 in 20 so refolding directly from denatured reduced protein in GuHCl or urea was not possible.

3.3 Analytical Techniques

3.3.1 Measurement of Protein Concentration

Lysozyme concentrations were determined spectrophotometrically using a Cecil Instruments Series 3000 spectrophotometer (Cecil Instruments, Milton Technical Centre, Cambridge) at 280 nm, using an extinction coefficient of 2.63 units ml/mg for native lysozyme and 2.37 units ml/mg for denatured lysozyme, path length = 1cm (Wetlaufer *et al.* 1974). These measurements

were complimented by measurements using the Coomassie protein assay (Biorad) using bovine carbonic anhydrase as the standard.

3.3.2 Lysozyme Activity Assay

Lysozyme is a bacteriolytic enzyme that is widely distributed in body fluids. Lysozyme cleaves the β-(1→4) glycosidic bonds of the murein cell wall of bacteria. This lysis results in the clarification of bacterial suspensions. These phenomena can be used to study lysozyme activity. By measuring the rate of clarification of a defined bacterial suspension by a sample of native lysozyme the specific activity of the native lysozyme can be calculated. The specific activity of lysozyme is defined as the decrease in absorbance units per mg of lysozyme per unit time under defined conditions. In the same way the specific activity of a solution of refolding lysozyme can be calculated. Yield is defined as the specific activity of the refolding lysozyme divided by the specific activity of native lysozyme under identical conditions.

$$\text{Yield (\%)} = \frac{\text{specific activity of refolded lysozyme}}{\text{specific activity of native lysozyme}} \qquad \textbf{Equation 3-1}$$

Lysozyme activity was determined at 25 °C by following the decrease in absorbance at 450 nm in a Cecil Instruments Series 3000 spectrophotometer (Cecil Instruments, Cambridge)of a 0.25 mg/ml suspension of *Micrococcus lysodeikticus* in 60 mM potassium phosphate buffer, pH 6.2. 100 µl of solution from the refolding buffer was added to 900 µl of *Micrococcus* suspension to give a final concentration of lysozyme of 0.0001 mg/ml (Morsky (1983)). A decrease in absorbance of 0.0026 units per minute corresponded to one unit of activity. In fed batch and continuous fed experiments the amount of refolding buffer added to the *Micrococcus* suspension was altered to give the same final concentration of lysozyme in the *Micrococcus* suspension. As previously stated lysozyme activities are reported as a percentage of the activity expected from the same molar concentration of native lysozyme. This

percentage is termed the yield of refolding and is equivalent to the specific activity of the refolded protein divided by the specific activity of native lysozyme under identical conditions. (Saxena and Wetlaufer (1970)).

The effect of the refolding buffer on the activity assay was investigated by adding identical volumes of refolding buffer to the assay mixture as would be used in a refolding experiment. The addition of refolding buffer to the *micrococcus* solution had negligible effect on the absorbance measured at 450 nm.

The effect of the chemicals in the refolding buffer on the assay of the native lysozyme was investigated. In all cases no aggregation was observed at 450 nm and the activity of native lysozyme in refolding buffer was identical to that in 0.1 M Tris pH 8.

3.3.3 Verification of the full reduction of di-sulphide bonds. (Ellman Assay)

Verification of the full reduction of lysozyme was achieved using the Ellman assay. (Ellman (1959)) The assay measures the nitrobenzoate released upon the reaction of a thiol group (-SH) with dinitrothiobenzoate (DTNB) and is used to ensure that the protein is fully reduced. 50 µl of 3mM DTNB solution (0.1 molar acetic acid pH 7.3) was added to 1 ml of denatured reduced lysozyme solution (0.1 mg/ml protein 0.1 M acetic acid, 1mM EDTA). The increase in absorbance was then measured at 412 nm in a spectrophotometer (Cecil Instruments, Cambridge). From the increase in absorbance the molar concentration of thiols can be calculated (E^{412}=13700/M if GuHCl is present, 14150/M cm in its absence) From this data it was calculated that using the denaturation methods described that there are 8 free thiols in the denatured reduced lysozyme protein verifying that the protein is fully reduced.

3.3.4 Measurement of Aggregation

Aggregation of refolding proteins is a complex phenomenon. The reaction can be compared to polymerisation where a multitude of reactions between different sized polymers occur simultaneously. It is extremely difficult to measure specific rate constants for each of these reactions. Quantitative data however can be obtained spectrophotometrically by measuring turbidity. As aggregation in a system increases the turbidity of the system increases. This method can be used to measure the extent of aggregation for different refolding experiments.

Batch refolding experiments were performed and the increase in turbidity caused by aggregation was measured at 450 nm in a Cecil Instruments Series 3000 spectrophotometer. Renaturation was initiated by adding 1 ml of a 20 mg/ml solution of denatured reduced lysozyme to 200 ml of renaturation buffer to give a final concentration of 0.1mg/ml of lysozyme (0.1M Tris-HCl, pH8.2, 1 mM EDTA, 3mM reduced glutathione and 0.3 mM oxidised glutathione). This corresponds to a dilution factor of 200. The renaturation solution was incubated in a water bath at the desired temperature and the turbidity was measured at several time points. The aggregation reaction is too rapid to gain any useful kinetic data from batch experiments by this method but it can be used to monitor the degree of aggregation in both fed-batch and continuous refolding experiments.

To assess the applicability of this method for studying aggregation under different conditions a series of batch refolding experiments were performed. Renaturation was initiated by adding volumes of 10, 15 and 20 mg/ml of denatured reduced protein to 200 ml of renaturation buffer (0.1M Tris-HCl, pH8.2, 1 mM EDTA, 3mM reduced glutathione and 0.3 mM oxidised glutathione) to give different final concentrations of lysozyme. The renaturation solution was incubated in a water bath at 25°C and 1ml aliquots were measured in the spectrophotometer at 450 nm.

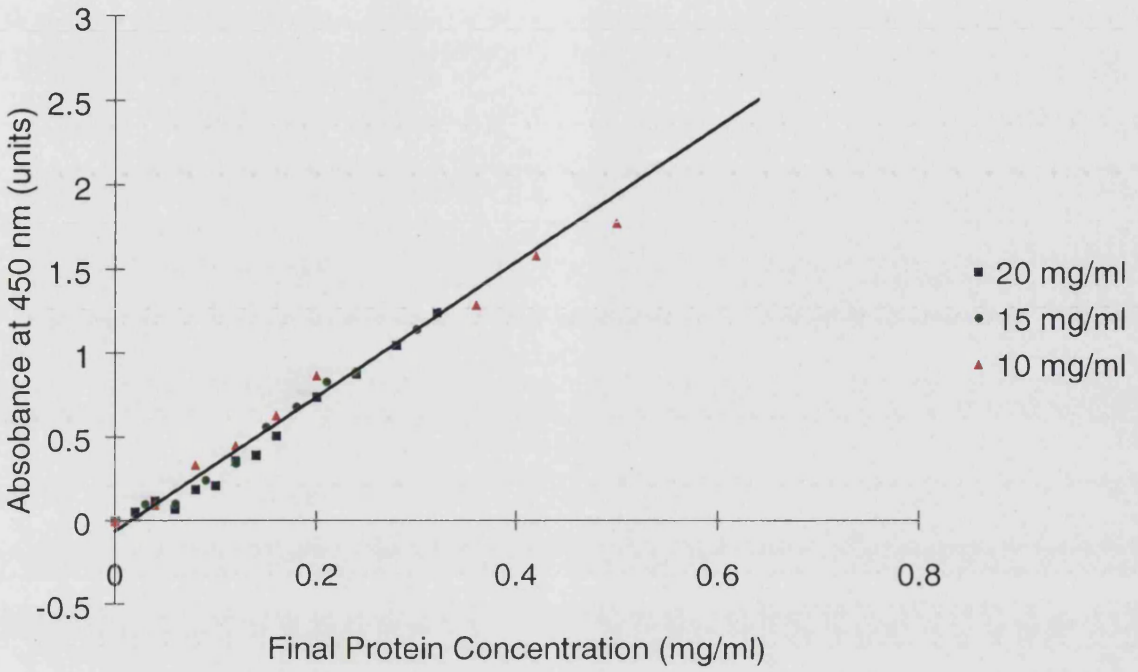

Figure 3-2 The relationship between absorbance and lysozyme aggregation during refolding.

As can be seen from Figure 3-2, despite different initial conditions of refolding the relationship between the final concentration of lysozyme in the refolding buffer and absorbance is linear and approximately equal in all three cases. This indicates that under different conditions the relationship between absorbance and aggregation is similar. Therefore this technique is valid for directly comparing the extent of aggregation in different experiments.

3.4 Reproducibility

The results for a typical experiment are shown in Figure 3-3. All experiments were performed a minimum of three times. The results presented throughout the thesis are the mean of three experiments.

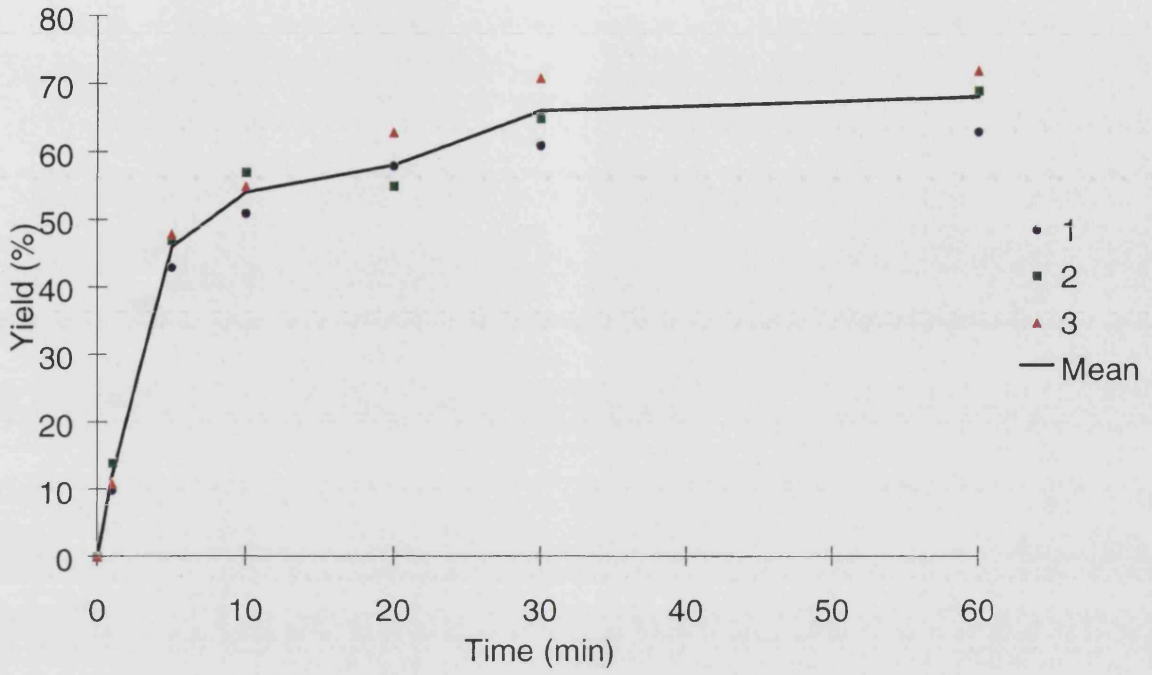

Figure 3-3 The variance of data for the refolding of lysozyme from 6M GuHCl 0.15M DTT into refolding buffer at 0.015 mg/ml lysozyme.

Figure 3-3 shows the variance of data for the refolding of lysozyme from 6 M GuHCl, 0.15M DTT into refolding buffer at 0.015 mg/ml lysozyme. Three identical experiments were performed and are represented by the numbers 1-3, the mean of the results is given by the dark line running through the points.

The standard deviation of the results was calculated using the following expression.

$$\sigma^2 = \frac{1}{n-1}\sum_{i=1}^{n}(x_i - \bar{x})^2$$ **Equation 3-2**

where σ^2 is the standard deviation, n is the number of samples, x_i is the i^{th} sample and $\bar{x}$ is the arithmetic mean of the samples. The percentage

variance is calculated by dividing the standard deviation by the arithmetic mean and multiplying by 100. The percentage variance makes comparisons between the standard deviations of different series easier.

Table 3 Results for the refolding of lysozyme from 6M GuHCl 0.15M DTT into refolding buffer at 0.015 mg/ml. Calculation of the mean, standard deviation and percentage variance

Time (min)	Sample 1 Yield (%)	Sample 2 Yield (%)	Sample 3 Yield (%)	Mean Yield (%)	Standard Deviation	Percentage Variance
0	0	0	0	0	0	0
1	10	14	11	12	1.7	14.2
5	43	47	48	46	2.2	4.7
10	51	57	55	54	2.5	4.6
20	58	55	63	58	3.3	5.7
30	61	65	71	66	4.1	6.2
60	63	69	72	68	3.7	5.5

As can be seen in Table 3 the data at 1 minute has a high variance, 14.2%. This is likely to be due to the fact that during the first minute that the rate of refolding is greatest and it is the time at which the activity is changing most rapidly. Therefore slight differences in the sampling time will make large differences in the observed yield.. After the first minute the variance settles to approximately 5%. This is typical of all refolding experiments

Note: When unfolded lysozyme was added to the refolding buffer during batch refolding experiments the system was not agitated. It is noted that this may lead to inaccuracies in the results due to a persons inability to accurately pipette a sample into a beaker of fluid repetitively. This stems from the different mixing in the system depending on the method of addition. The method of addition will affect both macro- and micro-mixing. It is beyond the scope of this text to write a discussion on the diffusivities of various molecules and the effect thereof of the type of mixing used upon their diffusion through

an aqueous medium. The above analysis has shown that the process is reproducible.

4. The Effect of System Variables on the Batch Refolding of Lysozyme

Batch experiments were carried out to discover which are the most significant variables in the refolding of lysozyme. The effects of the type of denaturant, protein concentration, initial protein concentration/dilution factor, pH, residual guanidine hydrochloride concentration, residual dithiothreitol concentration and temperature are investigated. It was found that the effects of these parameters are related and interdependent. Therefore, a certain degree of cross referencing within the Chapter is required.

These experiments also yield useful kinetic data which can be used to model the system being studied.

4.1 The effect of the type of denaturant on the refolding of lysozyme

It has been shown for the refolding of several proteins that the type of denaturant used has an effect on the refolding yield obtained. Kotik *et al.* (1995) have compared the refolding of lysozyme from dimethyl sulphoxide and GuHCl. In this section a comparison is made between the refolding of lysozyme from GuHCl, urea and acetic acid.

Various quantities of 10 mg/ml denatured reduced lysozyme in 6M GuHCl and 0.15M DTT or 8M urea or 0.1 M acetic acid were added to 200 ml of refolding buffer at 40 °C to give the desired final concentration of lysozyme. Refolding was studied in the range of 0.15 mg/ml to 0.2 mg/ml of lysozyme in the refolding buffer.

The effect of increasing the concentration of denatured lysozyme and the type of denaturant used in the refolding buffer on the yield of active lysozyme can be seen in Figure 4-1.

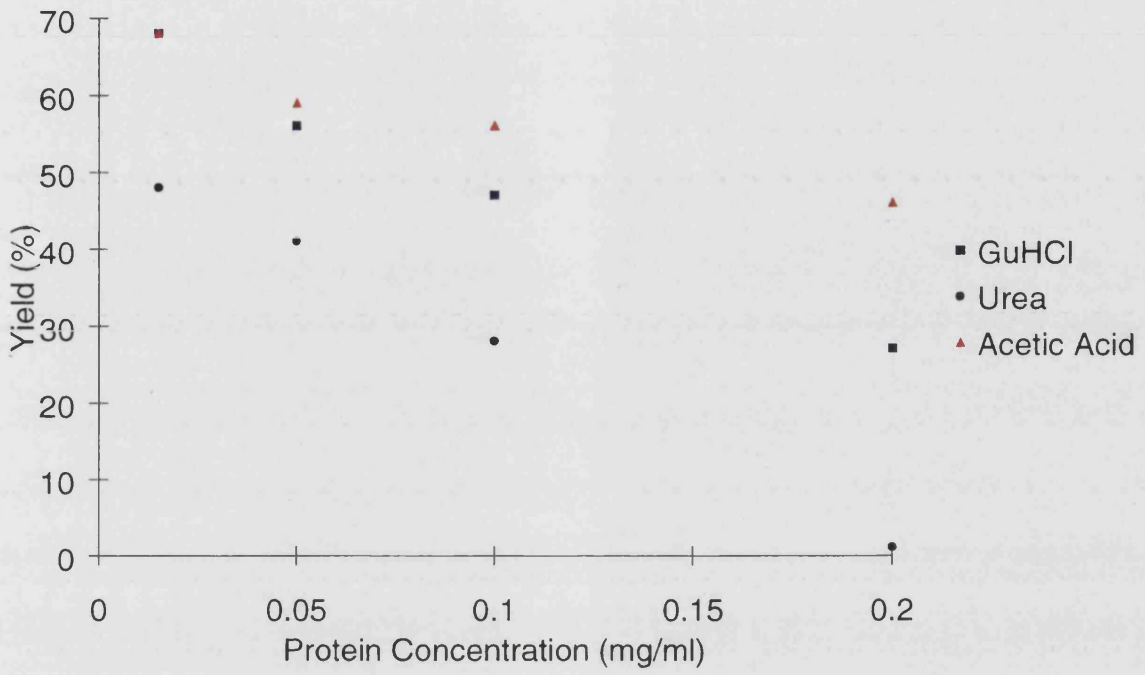

Figure 4-1 The effect of type of denaturant on the refolding yield of lysozyme.

Figure 4-1 shows the effect of type of denaturant on the refolding yield of lysozyme. In all three cases as the concentration of lysozyme in the refolding buffer is increased the yield of refolded native lysozyme is reduced. The maximum yield achieved is similar in both the refolding from acetic acid and from GuHCl (68%). The maximum yield obtained for the refolding of lysozyme from urea was 49%. When refolding from urea and from GuHCl there is a significant decrease in the observed yield as the concentration of the protein in the buffer is increased. In the case of urea the yield falls from 49% to practically zero and over the same range the yield of refolding falls from 68% to 27%. The loss in yield is lower for the refolding from acetic acid the yield falls from 68% to 50%. The time course data for the above experiments can be found in the Appendix: Section 11.3.

The difference in the yield of refolded lysozyme at low lysozyme concentration when refolding from different denaturants is thought to be due the effect of

the low residual concentration of the denaturants in the refolding buffer and the state of the denatured molecule prior to refolding. Denatured reduced lysozyme in 0.1M acetic acid has a more compact structure than denatured reduced lysozyme in either 6M GuHCl 0.15m DTT or 8M urea 0.15M DTT (Saxena and Wetlaufer (1970)). Therefore when the denatured reduced lysozyme in acetic acid is diluted into refolding buffer it already has a more stable structure than the reduced denatured lysozyme in GuHCl or urea. This in turn leads to higher refolding yields. It is proposed that the refolding yield of GuHCl is higher than the refolding yield of urea because of the ionic nature of the GuHCl. At low concentrations this will stabilise the refolding intermediates formed. Matsubara *et al.* (1993) showed that the kinetics of refolding of lysozyme were inhibited by increasing concentrations of urea in the refolding buffer. Over ninety minutes increasing urea concentration decreased the yield of refolded lysozyme.

The decrease in yield observed as the concentration of refolding lysozyme in the refolding buffer is increased is due to a competitive aggregation reaction (See Section 4.6). The reason for the greater loss of activity when refolding from GuHCl and urea is due to increasing concentrations of denaturant and reducing agent in the refolding buffer. For the refolding of lysozyme from GuHCl it has been shown that the protein refolds to a compact native like state (molten globule) within 4 ms (Chaffotte *et al.* (1992)). Fluorescence experiments by Denton *et al.* (1994) suggest that this structure is less compact in increasing concentrations of GuHCl in the refolding buffer. Therefore the molecule is less stable and more likely to aggregate. This hypothesis is supported by results in Sections 4.3 and 4.4.

It has been shown that for the refolding of lysozyme it is best to refold from denatured reduced protein in 0.1M acetic acid. Although at low concentrations of lysozyme yields from GuHCl are the same as from acetic acid, as the protein concentration is increased there is greater loss in activity

than when refolding from 0.1M acetic acid. This is thought to be due to the increasing concentration of reducing agent and GuHCl in the refolding buffer. Refolding from urea gives the lowest yields. It is thought that urea stabilises the molten globule and reduces the kinetics of refolding. Refolding from urea may prove more effective if the length of the experiment were increased.

4.2 The effect of pH on the refolding of lysozyme

Native lysozyme is stable over a wide range of pH values. The pH can be reduced to 2 at 0.1M salt concentration before any significant structural changes occur. This is unusual as lysozyme is an extremely basic protein. The effect of pH on the refolding protein is likely to be more pronounced. pH governs the reducing potential of the refolding buffer and will significantly affect di-sulphide formation. Saxena and Wetlaufer (1970) studied the effect of pH on the refolding of lysozyme in the limited range of pH 7.4 to pH 8.6. In this study the effect of pH on refolding of lysozyme is investigated over a wide range of values..

200 µl of 10 mg/ml denatured reduced lysozyme in 0.1M acetic acid was diluted 1000 times into 200ml of 0.1M Tris HCl 3mM reduced glutathione 0.3 mM oxidised glutathione at different pH's at 40 °C to give a final concentration of 0.01 mg/ml of lysozyme. Figure 4-2 and Figure 4-3 show the effect of the pH on the refolding yield of lysozyme.

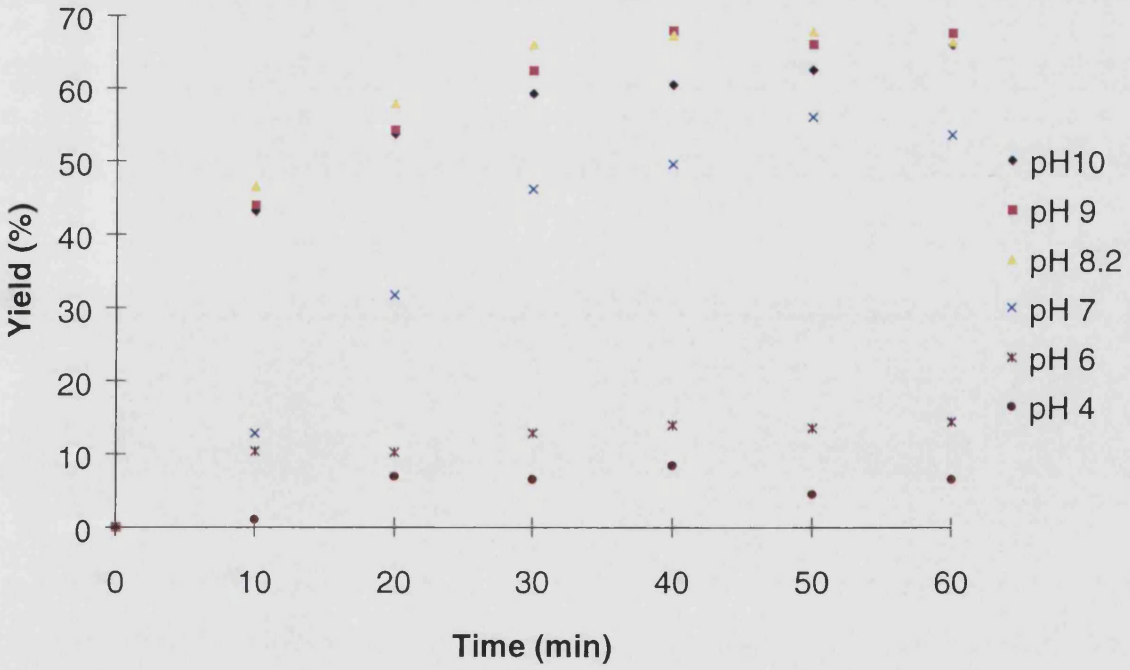

Figure 4-2 The effect of pH on the refolding yield of lysozyme.

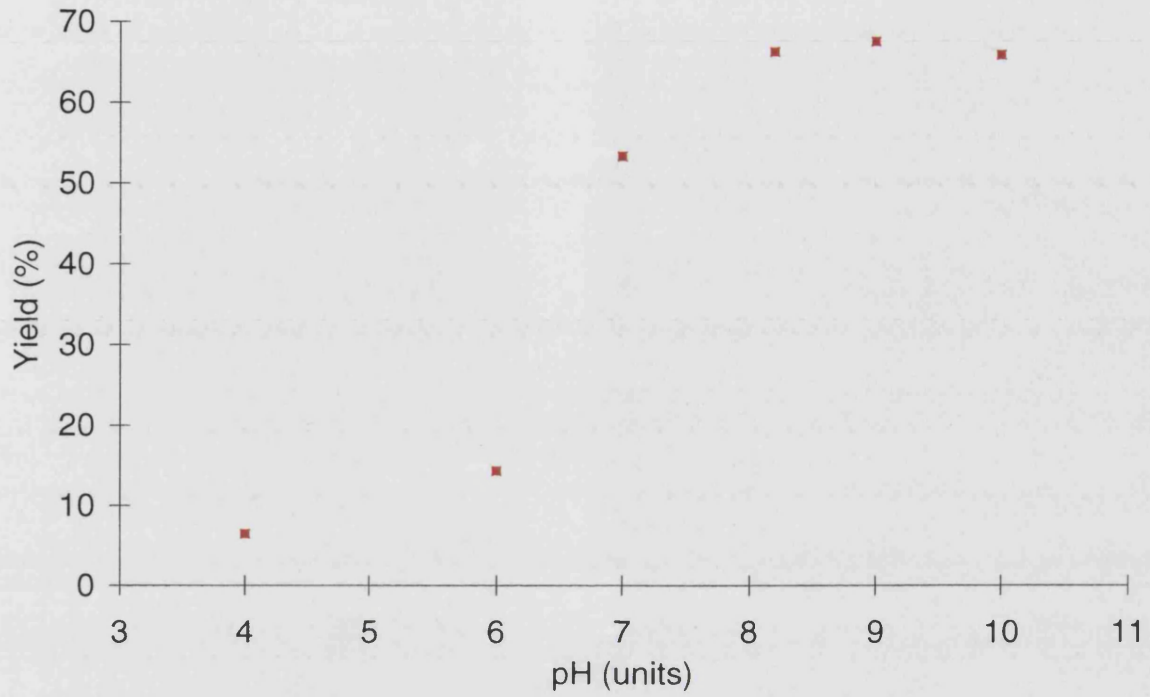

Figure 4-3 The effect of pH on the final yield of refolding.

pH affects both the final yield and rate of refolding. The final yield of refolded protein increases in a sigmoidal manner as pH increases. The yield increases from 7% at pH 4 to a maximum yield of approximately 70% between pH 8 and 9 which is followed by a slight decrease in yield at pH 10. The observed rate of refolding also increases as the pH increases.

Below a pH of seven it is likely that the reducing potential of the refolding buffer is too low to allow the correct formation of di-sulphide bonds (Creighton and Goldenberg (1986)). Also, at low pH, anions of salts bind to the protein and reduce the net solubility of the protein (Bell *et al.* (1993)). This would explain the low yield at low pH.

Between pH 8 and pH 9 a high yield is obtained. As lysozyme is a basic protein it is likely that refolding intermediates will be more stable under alkaline conditions than acidic ones. Under these conditions oxido-shuffling

of di-sulphide bonds via the mixed di-sulphide exchange system will be at an optimum (Saxena and Wetlaufer (1970)).

Proteins are generally least soluble at their isoelectric point. The isoelectric point of lysozyme is 10.5 (Dobson *et al.* (1994)). This means that as the pH of the refolding buffer is increased from five to ten the solubility of the native protein is decreased. This could explain the slight drop in yield observed from pH 9 to pH 10

The effect of pH on refolding has been studied by a number of groups. Saxena and Wetlaufer (1970) studied the effect of pH on the refolding of lysozyme in the narrow range pH 7.4 to pH 8.6 and found that it had no effect on the yield of refolding. As can be seen from Figure 4-2 the yield and rate of refolding fall dramatically below a pH of 7. Similar results have been observed for the refolding of tissue plasminogen activator (Rudolph *et al.* (1990)).

From these results and the work published it can be seen that the refolding of proteins containing di-sulphide bonds occurs under alkaline conditions. For the refolding of lysozyme and other proteins studied there is usually a plateau region where changing the pH has very little effect on refolding. This plateau has been found to be between 1 and 2 pH units. This is significant with respect to refolding on an industrial scale. As is shown is sections 4.3 and 4.4 low concentrations of denaturant or reducing agent in the refolding buffer greatly reduce the obtained yield of native lysozyme. Urea, GuHCl and DTT also represent high material costs. If inclusion bodies can be denatured at high protein concentration this minimises the amount of denaturant used. The denaturant can then be replaced by acetic acid which is cheaper than urea or GuHCl and when the denatured material is added to the refolding buffer only the pH will change. There will be no increase denaturant or reducing agent concentration and with respect to refolding the solvent conditions will remain constant. This especially important when

contemplating fed-batch or continuously fed refolding systems (See Sections 5.4.1 and 5.4.2).

4.3 The effect of residual GuHCl concentration on the refolding of lysozyme.

When refolding reduced denatured protein from 6M GuHCl and 0.15M DTT into refolding buffer there will be a residual concentration of denaturant and reducing agent present in the refolding buffer. The experiments in sections 4.1 and 4.7 have suggested that this residual concentration may have a significant effect on the refolding of lysozyme..

200 µl of 10 mg/ml denatured reduced lysozyme in 0.1M acetic acid was diluted 1000 times into 200ml of 0.1M Tris HCl 3mM reduced glutathione 0.3 mM oxidised glutathione pH 8.2 at 40 °C to give a final concentration of 0.01 mg/ml of lysozyme. GuHCl was added to the renaturation buffer to give the desired concentration of denaturant. Figure 4-4 shows the effect of the residual GuHCl concentration in the refolding buffer on the refolding yield of lysozyme

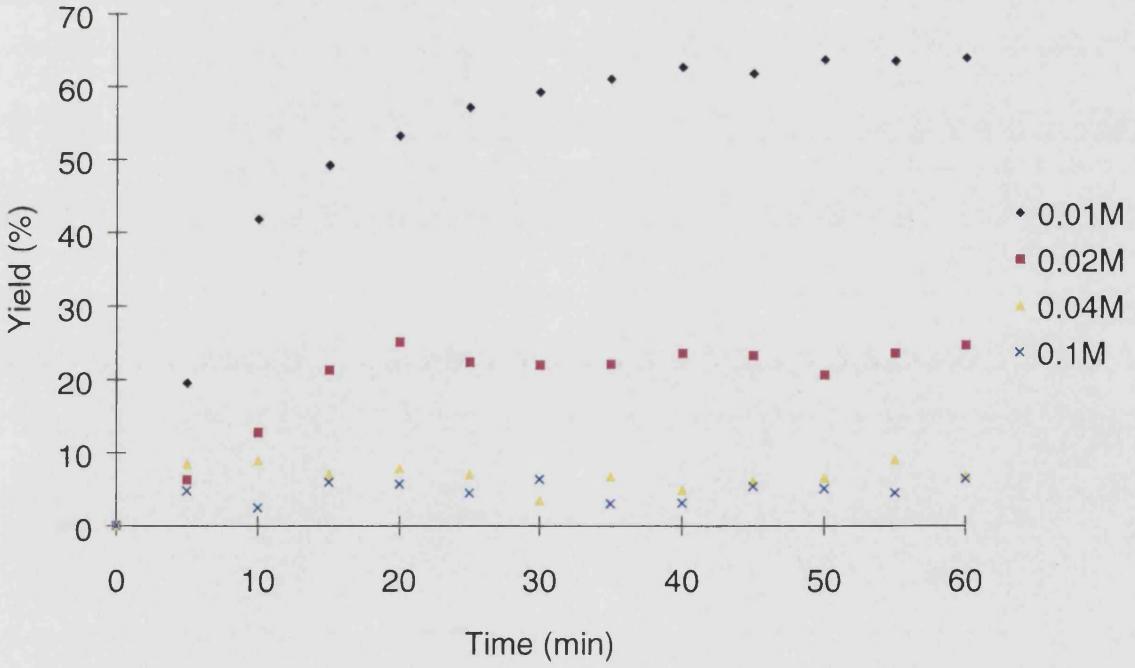

Figure 4-4 The effect of residual GuHCl concentration on the refolding of lysozyme

The refolding of denatured reduced lysozyme is extremely sensitive to the concentration of denaturant in the refolding buffer. At 0.02M GuHCl the refolding of lysozyme is strongly inhibited and at 0.04 M there is practically no regain of activity. In all experiments no aggregates could be detected at 450nm.

As shown above, the final denaturant concentration has a significant effect on the refolding of lysozyme. As the residual concentration of GuHCl is increased both the ionic strength and chaotropic nature of the molecule are increased. The concentration of denaturant in and the ionic strength of a solution of refolding protein determines which refolding intermediates proliferate. During denaturation GuHCl binds to hydrophobic residues and exposes the hydrophobic core of the protein (Horowitz and Criscimagna (1986)). These combined effects may lead to an increase in unproductive intermolecular associations which would explain the decrease in yield observed. However, as in all experiments no aggregates could be detected at 450nm, even at 0.1M GuHCl when less than a 10% yield is achieved, aggregation does not increase significantly, the increase in ionic strength of the refolding buffer is not likely to be responsible for the observed loss in yield

A more likely explanation for the observed loss in yield is the effect of the denaturant on the active site of the protein. Lysozyme can be alikened to an ellipsoid cleft down its longest axis and joined at one end (See Appendix: Section 11.1). The active site of an enzyme is extremely sensitive and during the denaturation of a number of enzymes it has been observed that inactivation occurs at lower concentration of denaturant than denaturation (Tsou (1986)). The enzymes which have been identified to be inactivated prior to unfolding include creatine kinase, glyceraldehyde-3-phosphate dehydrogenase, ribonuclease A and lactic dehydrogenase. (Tsou (1993)). This may explain the observed decrease in yield obtained as the concentration of the denaturant is increased. The low concentration of denaturant may well

disrupt the active site of lysozyme enough to reduce the yield of active protein. The active site of the lysozyme is buried in the cleft close to the hydrophobic core of the protein. The active site consists primarily of two amino acids, Glu 35 and Asp 52. Asp is negatively charged and would be expected to interact with the positively charged Gdm^+ ion. This association and the association of Gdm^+ with hydrophobic residues close to the active site would explain the decrease in yield as the residual concentration of GuHCl is increased. Native lysozyme activity is unaffected by low concentrations of GuHCl (See Appendix: Section 11.4). However as shown in Section 4.2, the sensitivity of the refolding molecule to environmental parameters is much higher than the native structure. This is because residues normally protected from interactions with chemicals in the bulk phase become exposed during denaturation (London *et al.* (1974)). This theory is supported by the work of Denton *et al.* (1994) who showed that increasing the concentration of GuHCl in the refolding buffer increased the size of lysozyme refolding intermediates and is consistent with the observation that although there is a decrease in yield there is no observed increase in aggregation.

Several studies have examined the effect of residual denaturant concentration on refolding. Damodaran (1987) found that the refolding of bovine serum albumin was inhibited when 2.0 M urea was present in the refolding buffer. Rudolph *et al.* (1979) showed that residual denaturant concentration has a detrimental effect on the recovery of activity during the refolding of lactic dehydrogenase. Horowitz and Criscimagna (1986) used CD and fluorescence measurements to show that low concentrations of GuHCl expose apolar residues and reduce the yield of refolded rhodanese. Cleland and Wang performed an extensive study of the effect of GuHCl on the refolding and aggregation of carbonic anhydrase B (Cleland and Wang (1990)). In contradiction to these studies the refolding of chymotrypsinogen was strongly

enhanced by the addition of 1.0M GuHCl or 2.0M urea (Orsini and Goldberg (1978)).

When refolding proteins by dilution the level of denaturant in the refolding buffer has to be reduced to a level which allows refolding to occur. The optimum concentration of GuHCl in the refolding buffer is protein specific and depends on the refolding intermediates formed at that concentration. For the refolding of lysozyme it has been shown that refolding is optimal at low concentrations of GuHCl. At 0.01 mg/ml the loss in activity is not due to an increase in aggregation but is probably due to inactivation of the protein. These results support the theory that the observed decrease in activity when refolding protein from different concentrations of denatured reduced protein is due to increasing denaturant concentration (Sections 4.1 and 4.7). They also suggest that for the refolding of lysozyme that the denaturant should be removed from the refolding system completely.

4.4 The effect of dithiothreitol on the refolding of lysozyme.

Dithiothreitol is a powerful thiol reducing agent. It is a cyclic molecule and strongly affects thiol groups on proteins (Cleland (1964)). The experiments in Section 4.7 have suggested that this residual concentration may have a significant effect on the refolding of lysozyme.

200 µl of 10 mg/ml denatured reduced lysozyme in 0.1M acetic acid was diluted 1000 times into 200ml of 0.1M Tris HCl, 3mM reduced glutathione, 0.3 mM oxidised glutathione, pH 8.2 at 40 °C to give a final concentration of 0.01 mg/ml of lysozyme. DTT was added to the renaturation buffer to give the desired concentration of reducing agent. Figure 4-5 shows the effect of the DTT concentration on the refolding of lysozyme.

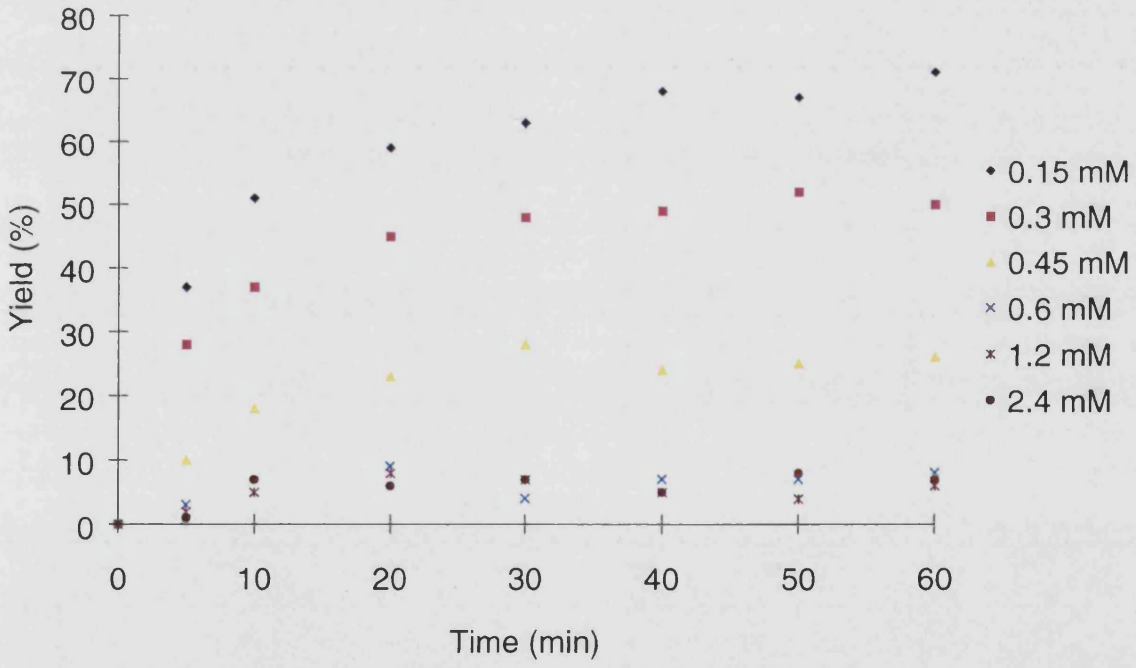

Figure 4-5 The effect of DTT concentration on the refolding of lysozyme

Refolding is highest at low concentrations of DTT and falls rapidly to zero reactivation at concentrations above 0.6 mM. This is probably due to the shift in the redox potential of the system. As the concentration of DTT is increased

the refolding buffer becomes a more reducing environment, di-sulphide bonds will not reform properly under these conditions and therefore less activity is recovered.

Adding DTT to the system shifts the equilibrium of reducing and oxidising agent. In this experiment a ration of 3 mM reduced glutathione to 0.3 mM oxidised glutathione is used. There is practically no refolding above 0.6 mM DTT. This approximates to a change in the molar redox ratio of reduced and oxidised thiol in the system from 10:1 to 3:1. The loss in activity is consistent with the results of Saxena and Wetlaufer (1970) who found that at a concentration of 3mM reduced glutathione and a redox ratio of three to one that no activity was regained.

Although the effect of DTT on the refolding of proteins has not been studied in detail the effect of other thiol agents such as reduced and oxidised glutathione (GSH and GSSG) has been (Saxena and Wetlaufer (1970)). Bradshaw *et al.* (1967) studied the effect of cystine on the refolding of lysozyme and were one of the first groups to show that refolding of proteins with di-sulphide bonds proceeds via di-sulphide shuffling. Saxena. and Wetlaufer (1970) found that regeneration of active lysozyme is highest at a 10:1 molar ratio of reduced glutathione to oxidised glutathione and when the concentration of reduced glutathione is $5x10^{-3}$ M. It may seem odd at first that optimal refolding occurs under reducing conditions. However *in vivo* folding generally occurs under reducing conditions. Protein di-sulphides are generally more stable than non-cyclic thiol groups. A reducing system containing both reducing and oxidising agents allows mis-formed unstable di-sulphides to break and reform properly. Once formed correctly the reducing potential of the system is not high enough to reduce the stable bond. The concentration of glutathione in mammalian tissue has been studied by Tietze (1969). It was found the glutathione existed at milli-molar concentrations and in ratios of GSSG:GSH of between 20:1 and 100:1.

In denaturing lysozyme, 0.15M DTT has been used to fully reduce the di-sulphide bonds. In an industrial process in would be worth investigating the minimum amount of DTT needed to fully reduce the protein in question. If the concentration of DTT can be minimised at this stage then removal of the reducing agent may not be necessary.

It has been shown that residual DTT has an adverse effect on the refolding yield off lysozyme. It is thought that this is due to the shift in the redox potential of the refolding buffer which in turn prevents the correct formation of di-sulphide bonds. These results support the theory that the observed decrease in activity when refolding protein from different concentrations of denatured reduced protein is due to increasing reducing agent concentration (Sections 4.1 and 4.7).

4.5 The temperature dependence of lysozyme refolding

200 µl of 10 mg/ml denatured reduced lysozyme in 0.1M acetic acid was diluted 1000 times into 200ml of 0.1M Tris HCl 3mM reduced glutathione 0.3 mM oxidised glutathione pH 8.2 to give a final concentration of 0.01 mg/ml of lysozyme. The refolding buffer was maintained at different temperatures using a water bath. Figure 4-6 shows the effect of the temperature on the refolding yield of lysozyme.

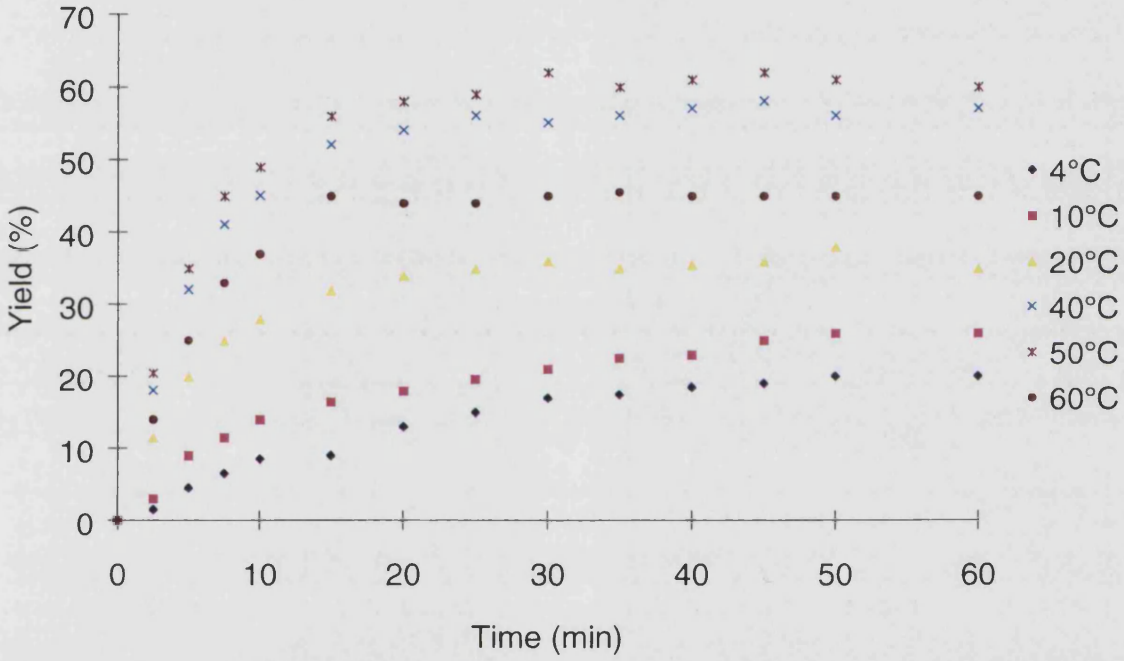

Figure 4-6 The effect of temperature on the refolding of lysozyme.

The refolding yield increases as temperature is increased from 4 °C to 50 °C and decreases again at 60°C. The solution was checked for aggregates by measuring the absorbance at 450 nm. The absorbance was zero so no aggregates large enough to scatter light were formed. Non-denaturing gel-electrophoresis was used to try to identify soluble aggregates; this technique rendered no useful data.

The majority of refolding experiments are performed in the range 0-40°C with room temperature (20-25°C) most commonly used . At temperatures well above this range the efficiency of folding of the proteins studied is decreased due to thermal denaturation (Fischer *et al.* (1993), Jaenicke and Rudolph (1989)). Studies of several proteins including rhodanese (Mendoza *et al.* (1991)), the dimeric form of Rubisco (Gatenby *et al.* (1990), Viitanen *et al.* (1990)), Fab fragments (Buchner and Rudolph (1991)) and the phage P22 tailspike protein (Seckler *et al.* (1989)) have shown that reactivation increases at low temperatures with a maximum at around 10°C. The increase in renaturation at lower temperatures is related to the reduction in the rate of aggregation allowing more protein molecules to reach the native state (Cleland and Wang (1990)).

It is thought that temperature directly affects the endothermic hydrophobic interactions which are thought to be the main driving force for refolding (Dill (1990)). Therefore an increase in temperature should lead to an increase in the rate of refolding. However an increase in temperature may also lead to an increase in denatured protein concentration and therefore an increase in aggregation. For each individual protein and given set of conditions a balance between these two phenomena must be found. If the thermally denatured molecules could be stabilised then refolding would be possible at higher temperatures. In the case of Rubisco it was found that efficient renaturation could be achieved at temperatures up to 37°C when refolded in the presence of chaperonins (Viitanen *et al.* (1990)).

The variance in the results in Figure 4-6 can be described by the Arhenius law.

$$k = k_0 e^{\frac{-E}{RT}} \qquad \text{Equation 4-1}$$

Where k is the specific rate constant (s^{-1}), R is the gas constant (J/mol K)=8.31, k_0 is the frequency factor (s^{-1}), T is temperature (K) and E is the activation energy (J/mol)

Rearranging Equation 4-1 gives

$$\ln k = \ln k_0 + \left(\frac{-E}{RT}\right) \qquad \text{Equation 4-2}$$

Plotting ln k versus 1/T gives ln k_0 as the intercept and -E/R as the gradient (Figure 4-8).

In order to determine the activation energy the rate constant for each temperature must be calculated. It is assumed that k is only a function of temperature and that refolding is a first order reaction.

For a first order reaction the rate of reaction, r_A, is given in Equation 4-3

$$r_A = C_{DO}\frac{dX_D}{dt} = -k\,C_D \qquad \text{Equation 4-3}$$

Where r_A is the rate of refolding mg/ml.s; X_D is the conversion of denatured protein; C_{D0} is the initial concentration of denatured protein (mg/ml) and C_D is the concentration of denatured protein at time t (mg/ml)

Conversion of denatured protein, X_D, at any given time can be defined as:

$$X_D = 1 - \frac{C_D}{C_O} \qquad \text{Equation 4-4}$$

Integrate and separate

$$-\ln(1 - X_D) = k\,t \qquad \text{Equation 4-5}$$

By plotting $\ln(1-X_D)$ against t for renaturation experiments at different temperatures the values of k_1 for each temperature can be calculated from the initial rates of reaction. Data up to 8 minutes is taken from Figure 4-6.

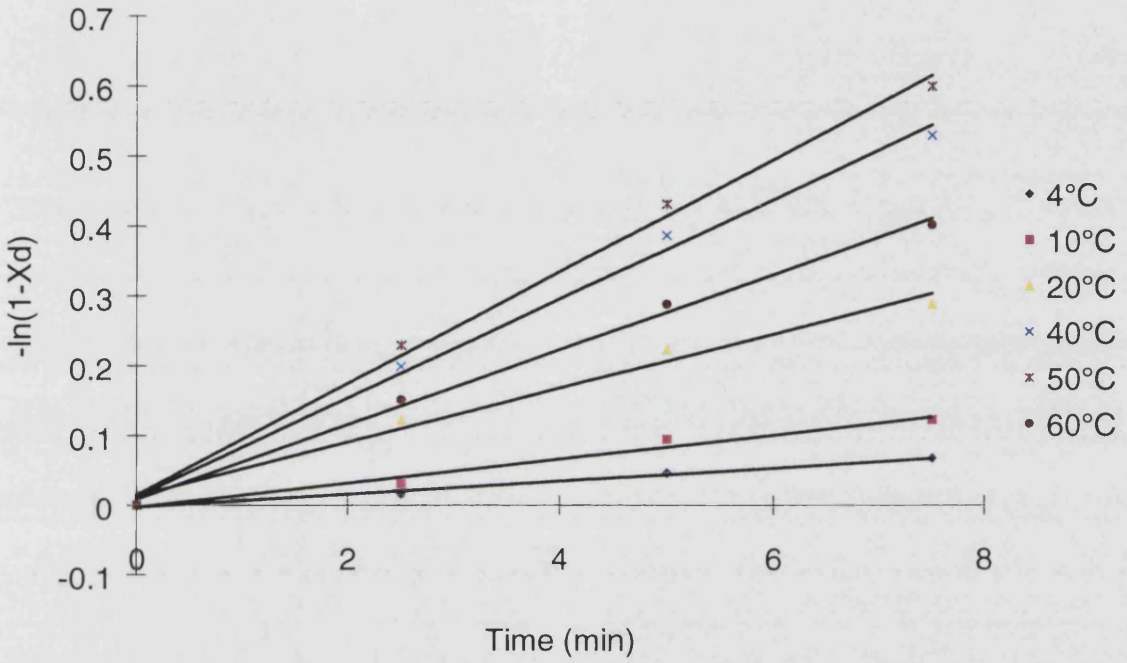

Figure 4-7 Calculation of the initial rates of refolding at different temperatures.

The data at 50 °C and 60 °C were not used in the calculation of the activation energy. These points did not fit into the expected linear plot for the Arhenius equation. At these temperatures it is likely that the protein starts to become thermally denatured (Jolles (1969) and Khechinashvilli *et al.* (1973)).

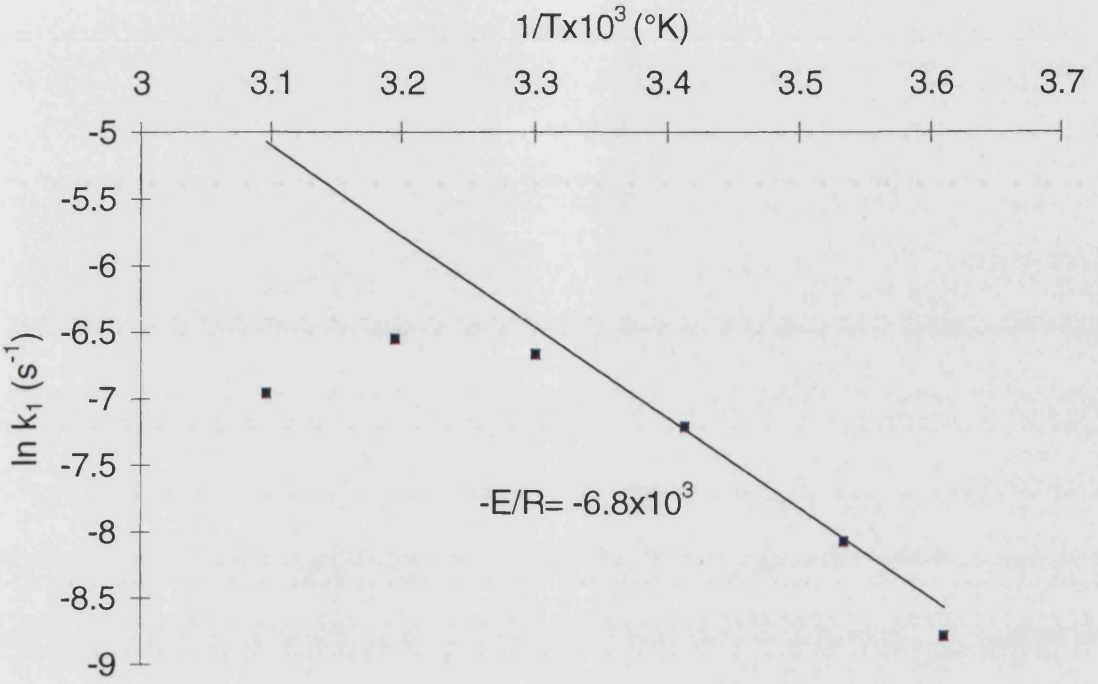

Figure 4-8 Arhenius plot for the refolding of lysozyme

$$-\frac{E(J/mol)}{R(J/mol.K)} = -6800(K^{-1}) \qquad \text{Equation 4-6}$$

$$E = 56(kJ/mol)$$

The calculated activation energy was approximately 56 kJ/mol. The linear regression was performed using the least squares method and the r^2 coefficient for the linear regression was 0.98. Privalov showed that the free energy of refolding for a small globular protein can be expected to be approximately 50 kJ/mol (Privalov (1992)). However the activation energy here is not a direct representation of the activation or free energy of refolding. The concentration of lysozyme in the refolding buffer for these experiments was 0.01 mg/ml. Even at this low concentration the refolding reaction is subject to a competitive aggregation reaction. The value calculated for the activation energy for refolding can be considered as an apparent activation

energy which takes into account the effect of temperature on the aggregation process as well as refolding.

It has been shown that temperature has a significant effect on the refolding of denatured reduced lysozyme. An increase in temperature from 4°C to 50°C was found to increase the yield of refolded protein from 20% to 64% respectively. 50 °C was found to be the optimum temperature for refolding. The reason for the increase in the yield is thought to be due to the endothermic nature of the refolding reaction. Using the Arhenius expression an apparent activation energy for refolding was calculated to be 56 kJ/mol. If refolding which exhibited the same temperature dependence of refolding as lysozyme were to be produced on an industrial scale the expense of maintaining a high temperature would need to be assessed with respect to the increased yield obtained

4.6 The effect of protein concentration on the refolding of lysozyme.

Protein concentration is well known for having a significant effect on the refolding of all proteins (Thatcher and Hitchcock (1994)).

Various quantities of 10 mg/ml denatured reduced lysozyme in 6M GuHCl and 0.15M DTT were added to 200 ml of refolding buffer at 40 °C to give the desired final concentration of lysozyme. The range of lysozyme concentration studied was 0.015 mg/ml to 0.2 mg/ml. The effect of increasing the concentration of denatured lysozyme in the refolding buffer on the yield of active protein can be seen in Figure 4-9.

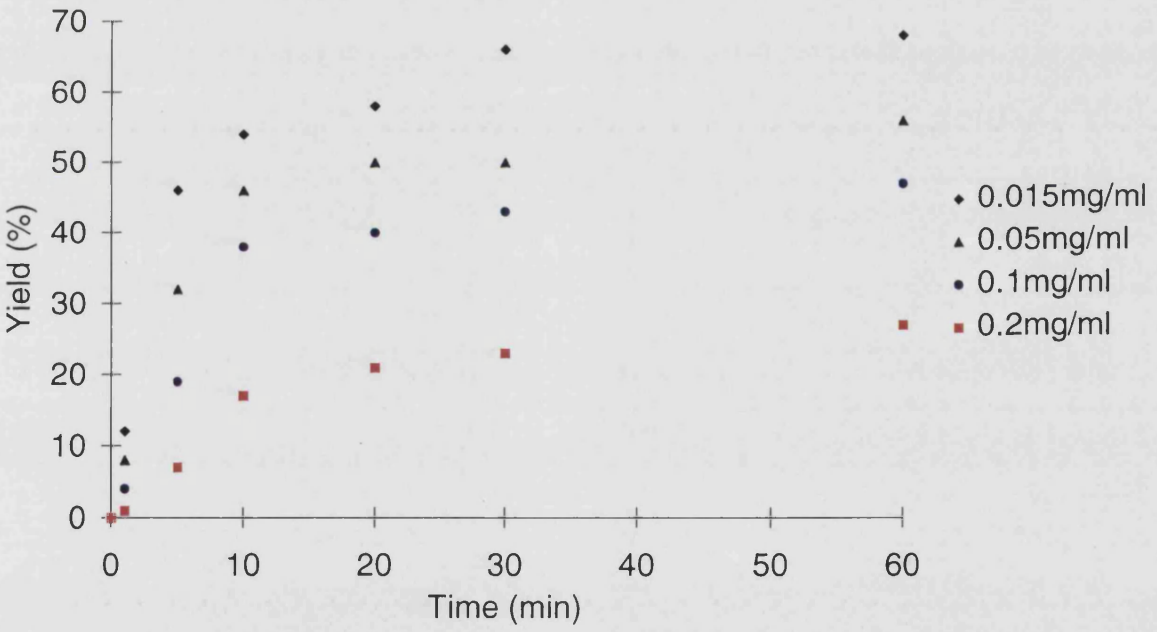

Figure 4-9 : The effect of final protein concentration on the yield of refolded lysozyme.

As the amount of denatured reduced lysozyme added to the refolding buffer is increased from 0.015mg/ml to 0.2mg/ml the observed yield of active protein is decreased. The yield falls from 68% at 0.015 mg/ml to 28% at 0.2 mg/ml. The

decrease in yield can be attributed to aggregation of folding molecules. The turbidity of the refolding buffer increases from zero at 0.015 mg/ml to approximately 0.75 units (450 nm) at 0.2 mg/ml. The protein precipitates as it aggregates and at 0.2 mg/ml the white aggregates can be clearly seen.

In order to assess whether or not all non-active protein aggregated, the aggregates were collected using centrifugation, dissolved and the concentration of the dissolved material was measured. The aggregates in 1 ml of solution were collected using a micro-centrifuge and then re-dissolved in a 1ml of 6M GuHCl (0.1M Tris HCl pH8.2). The concentration of protein was then measured spectrophotometrically. Performing a mass balance on the protein added to the system it was calculated that any non-active protein aggregated (e.g. when refolding at 0.2 mg/ml a yield of 28% is obtained. The concentration of dissolved aggregates was approximately 0.14 mg/ml).

$$P_T = P_R + P_A \qquad \text{Equation 4-7}$$

$$P_R = P_T * Y \qquad \text{Equation 4-8}$$

where P_T is the total mass of protein added to the system, P_R is the mass of refolded protein, P_A is the mass of aggregated protein and Y is the yield if refolded protein.

The final concentration of refolded protein in the refolding buffer is often quoted as the most significant variable in the protein refolding process. As already stated, increasing the concentration of protein in a refolding system usually decreases the obtained yield (De Bernadez-Clark and Georgiou (1991)). The rate and final yield of active protein have been shown to be affected by protein concentration for several other proteins including carbonic anhydrase B (Cleland and Wang (1990)) and lactic dehydrogenase (Zettlemeissel *et al.* (1979)). Goldberg *et al.* (1991) performed refolding experiments with hen lysozyme but only achieved a maximum yield of 35%.

This is in contradiction to earlier work performed by Saxena and Wetlaufer (1970) who found similar results to those in Figure 4-9. The differences in the experimental method are in the method of dilution and the method of denaturation. Goldberg *et al.* (1991) refolded from 10 mg/ml lysozyme in 0.01M HCl into an identical refolding buffer to the one used by Saxena and Wetlaufer at 20 °C with a final concentration of 0.015 mg/ml and it is difficult to explain the large discrepancy in the reported figures. Goldberg *et al.* (1991) used vortex mixing to disperse the denatured protein in the refolding buffer which may lead to a lower refolding yield due to air-oxidation. Air oxidation refolding of reduced denatured protein has been shown to be less effective than the mixed di-sulphide method (Saxena and Wetlaufer (1970)). This is thought to be because di-sulphide exchange cannot occur during air oxidation (i.e. incorrectly formed di-sulphides do not break and reform correctly as is thought to happen in the mixed di-sulphide system). Despite obtaining a lower yield, Goldberg *et al.* (1991) calculated the half-life of reaction for lysozyme refolding to be 4.5 minutes which is similar to that observed in Figure 4-9. The kinetics of refolding are discussed more fully in Section 3.2.3.

Protein refolding is initiated by the removal of the denaturant, usually by dilution, which initiates the collapse of the unfolded molecule and the formation of secondary/early tertiary structure. The collapse of the molecule is thought to occur on a milli-second time-scale (Dobson *et al.* (1994), Chaffotte *et al.* (1992)). It is during this time that hydrophobic residues will become exposed and the protein will be most susceptible to aggregation (London *et al.* (1974), Horowitz and Criscimagna (1986)). If the protein concentration is high then non-productive reactions will predominate as hydrophobic regions are exposed and interact with exposed regions from other protein molecules. In the case of proteins with di-sulphide bonds, incorrect di-sulphide bond formation may also lead to aggregation (Shoemaker *et al.* (1985)). In dilute systems the aggregation reaction becomes diffusion limited and the refolding reaction predominates. To obtain a reasonable yield (i.e.

greater than 25%) refolding often has to be carried out at concentrations of less then 0.01 mg/ml. (Rudolph (1980), Rudolph *et al.* (1991))

In the case of bovine growth hormone it was found that although increasing the concentration of protein in the system did not reduce the final yield of refolded protein the rate of refolding was reduced (Brems *et al.* (1987)). The results were attributed to the formation on a transient polymer which readily dissociates to form the native protein. Similar results were obtained by Cleland and Wang (1992) for the refolding of carbonic anhydrase B. They showed that increasing protein concentration increased the concentration of an unstable dimer and that the rate of dissociation of the dimer was the rate limiting step in the refolding pathway. It is significant to note that both these studies were performed at low protein concentrations and that increasing the protein concentration beyond the range studied would probably result in increased aggregation and a lower yield.

Protein concentration affects all proteins in the same manner. Increasing the protein concentration in the refolding buffer decreases the yield of native protein. The magnitude of the effect is protein dependent. I have shown that increasing the concentration of lysozyme in the refolding buffer decreases the yield obtained. My results support and expand upon the work of Saxena and Wetlaufer (1970) and disagree with the results of Goldberg *et al.* (1991). As protein concentration affects all proteins in the same way this variable would provide an ideal basis for a generic approach for the optimisation of refolding.

4.7 The effect of initial protein concentration on the refolding of lysozyme

It has been suggested that the first few milliseconds are critical in the refolding of proteins (Dill (1990), Dobson *et al.* (1994), London *et al.* (1974), Chaffotte *et al.* (1992)). To investigate this the effect of the concentration of denatured reduced lysozyme prior to refolding is studied. This will give an idea of the importance of the protein concentration whilst the denatured reduced material is still mixing with the refolding buffer.

10 mg/ml denatured reduced lysozyme in 6M GuHCl , 0.15M DTT in 0.1M Tris HCl was diluted with 6M GuHCl , 0.15M DTT in 0.1M Tris HCl to give samples of 10, 5, 2 and 1 mg/ml of denatured reduced lysozyme. Different quantities of these solutions were added to 200 ml of refolding buffer at 40 °C to give a final concentration of 0.01 mg/ml of lysozyme. Figure 4-10 shows the effect of the initial protein concentration on the refolding of lysozyme

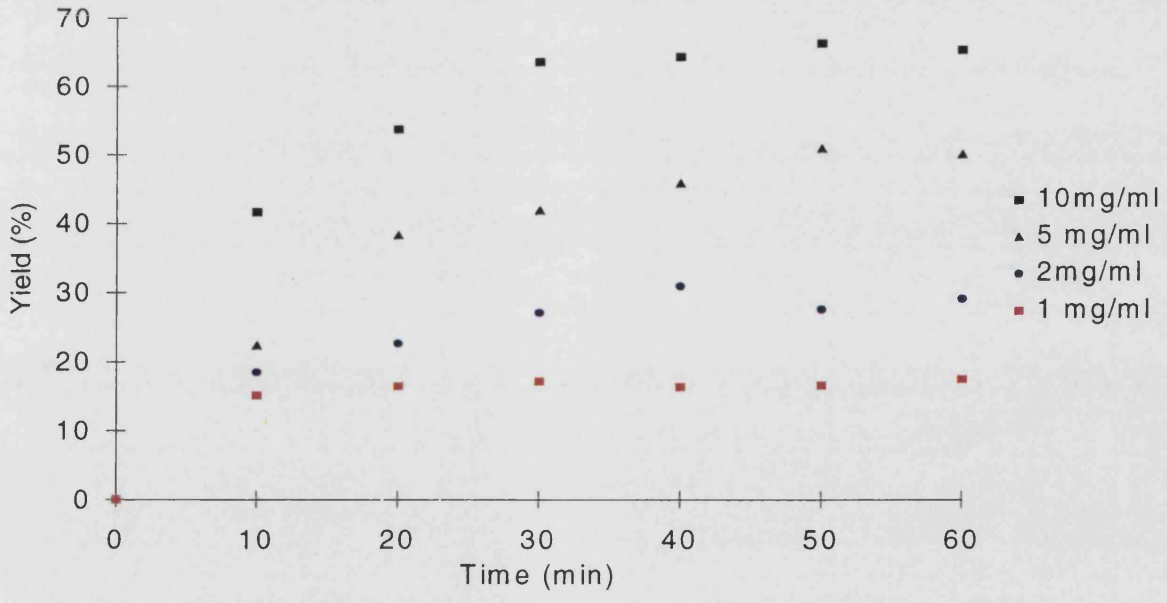

Figure 4-10 The effect of initial protein concentration on the refolding of lysozyme.

As the initial protein concentration was decreased from 10 mg/ml to 1 mg/ml the observed yield decreased from 65 % to 17 %. One would expect that as the initial concentration of the protein is decreased that the yield would increase as intermolecular reactions are reduced. However, as the dilution is decreased ten fold from 1 in 1000 to 1 in 100 the concentration of GuHCl and DTT in the refolding buffer increases from 6mM and 0.15 mM to 60 mM and 1.5 mM respectively.

These experiments suggest that both the residual denaturant concentration and the redox potential of the refolding buffer have a detrimental effect on the observed yield of refolding lysozyme. This is borne out by experiments performed into the effect of the solvent conditions on refolding yield. (See Sections 4.3 and 4.4)

When the effect of protein concentration on refolding is discussed, the topic is usually the final concentration of protein in the refolding buffer (i.e. refolded and aggregated). As Section 4.6 shows this is a significant variable, but the concentration of denatured protein and the solvent conditions prior to refolding are also significant. It is remarkable therefore to find numerous papers on refolding which do not quote this information, e.g. Buchner and Rudolph (1991) and Rudolph *et al.* (1992). A prime example is given by Fischer *et al.* (1992) who dilute denatured lysozyme "at least 50 times" to give a final protein concentration of 3.3 μg/ml. The first few milliseconds after addition of the denatured protein to the renaturation medium have been suggested to be the most critical (Goldberg *et al.* (1991). During this time, the protein is still undergoing mixing and the actual concentration of refolded protein will lie somewhere between a perfectly mixed system and the initial concentration of denatured protein. Therefore choosing the correct initial conditions is just as critical as choosing the correct final conditions. The initial conditions also dictate the final denaturant and reducing agent

concentrations both of which have been shown to be important factors in refolding. (See section 4.3 and section 4.4)

In order to investigate the effect of the initial protein concentration without the detrimental effects of GuHCl and DTT being present the following experiment was performed. 10 mg/ml denatured reduced lysozyme in 0.1M acetic acid was diluted with 0.1 M acetic acid to give samples of 10, 5, 2 and 1 mg/ml of denatured reduced lysozyme. Different quantities of these solutions were added to 200 ml of refolding buffer at 40 °C to give a final concentration of 0.01 mg/ml of lysozyme. The pH change due to the addition of different amounts of acetic acid was measured and found to be negligible. The results are shown in Figure 4-11

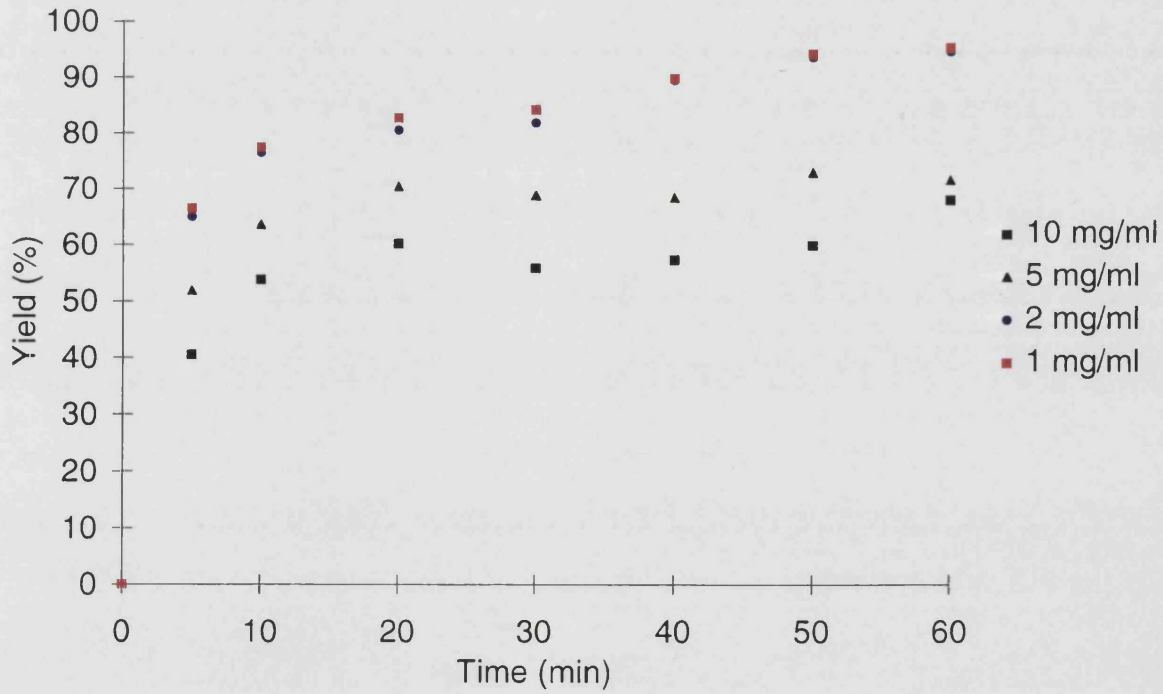

Figure 4-11 The effect of initial protein concentration on the refolding of lysozyme.

As the initial concentration of lysozyme is decreased the observed yield is increased from 68% to 95%. This is probably due to mass transfer limitations

affecting the aggregation process and allowing the refolding reaction to proceed unhindered. This proves that the first few seconds prior to reaching a perfectly mixed system are critical when considering a rational approach to refolding. This experiment was repeated for different final concentrations of lysozyme. The results are shown in Figure 4-12

A solution of 10 mg/ml denatured reduced lysozyme in 0.1M acetic acid was diluted with 0.1 M acetic to give samples of 10, 5, 2 and 1 mg/ml of denatured reduced lysozyme. Different quantities of these solutions were added to 200 ml of refolding buffer at 40 °C to give a final concentrations of between 0.01 mg/ml and 0.1 mg/ml lysozyme.

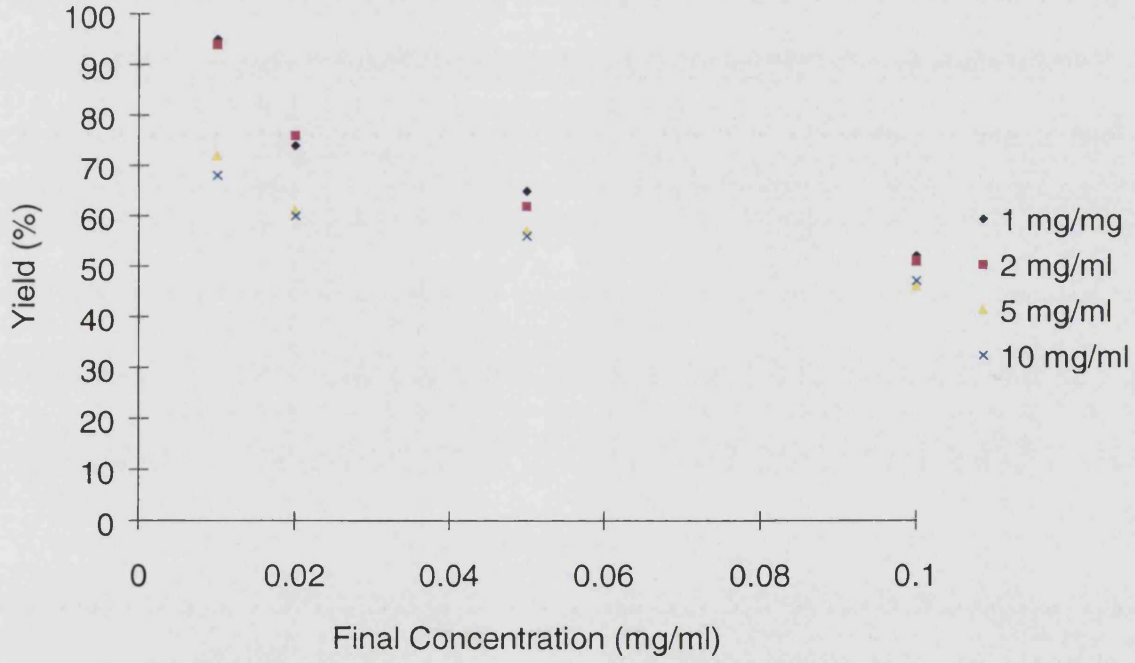

Figure 4-12 The effect of both the initial protein concentration and the final protein concentration on the refolding of lysozyme.

Figure 4-12 shows how the initial protein concentration affects the observed yield of refolded protein at different final concentrations of protein. The

initial protein concentration has the greatest effect on refolding when the final concentration of protein is low (0.01mg/ml). The effect is decreased as the final protein concentration is increased.

As the final concentration of protein is increased the effect of the initial concentration is less pronounced and at a final concentration of 0.1 mg/ml there is less than 10 % difference in the final yield observed The reason for the reduced difference in the effect of dilution at different concentrations of refolding lysozyme is probably due to the increased numbers of interactions between folding intermediates at high initial concentration of protein.

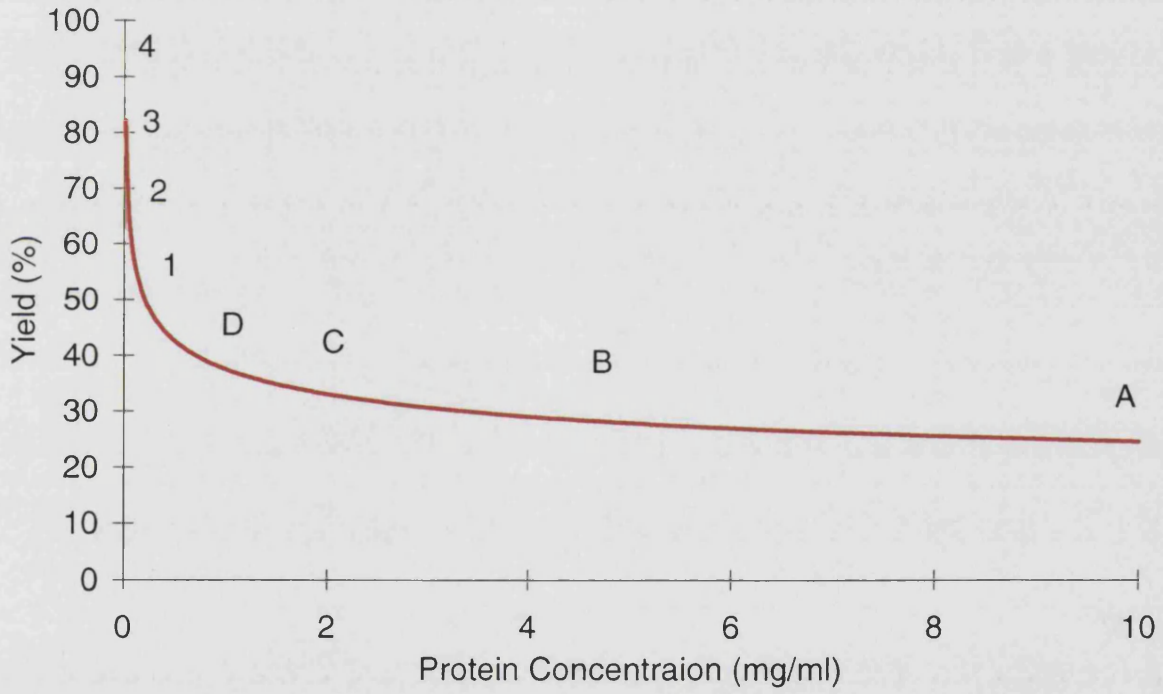

Figure 4-13 Explanation for observed difference in yield when refolding from different initial concentrations of denatured lysozyme

This theory is illustrated more clearly in Figure 4-13. The diagram indicates the yield obtained for a given final protein concentration. Letters A→D indicate the starting concentration of denatured lysozyme prior to refolding. Numbers 1→4 indicate the final concentration of refolded lysozyme in the

refolding buffer after refolding. The red line is a time line. The protein starts at a concentration represented by a letter (A→D) and then as the denatured concentrated solution mixes with the refolding buffer it is diluted until it reaches its new concentration represented by a number (1→4). Obviously a more concentrated solution (A) will take longer to reach equilibrium than a less concentrated solution (D) if the final concentration is the same in both cases.

The diagram shows that when refolding from 10mg/ml denatured reduced lysozyme that until the protein reaches the concentration of 1mg/ml aggregation predominates. The time taken for the denatured protein to move from concentration A to 4 (i.e. the mixing time) is significant with respect to aggregation. If the protein concentration in the denatured state is lower, position D, then the effect of mixing time is reduced because the time taken to attain a concentration where refolding predominates is less than when starting from denatured material at 10mg/ml, i.e. position A. Therefore a higher yield will be obtained when refolding from dilute denatured protein than when refolding from concentrated denatured protein.

The reason for the dilution effect being less pronounced when refolding at higher final protein concentration when the final yield is low can be explained as follows. When refolding to position 1 (0.1mg/ml) aggregation is extensive (50%) and the aggregation during mixing (i.e. moving from position A to 1 rather than D to 1) is insignificant with respect to the final yield. If the final concentration is low then it is more important to reach a protein concentration where aggregation is minimised quickly.

Goldberg *et al.* (1991) found similar results when refolding lysozyme at a final concentration of 0.05 mg/ml. They found that the renaturation yield was highest when refolding from 0.5 mg/ml (35%) and lowest when refolding from 20 mg/ml (10%). As mentioned earlier, the low yield observed by Goldberg *et al.* (1991) is probably due to the different refolding conditions used (Section 4.6). Although Goldberg *et al.* (1991) investigated this phenomena they only

performed the experiment using a single final concentration of 0.05 mg/ml and gave no clear explanation of their results.

The results show that the time taken to achieve a homogeneous solution is an important variable in refolding. The large difference in the observed yields due to the initial protein concentration is a significant result. This phenomena is not usually a variable which is investigated by researchers trying to optimise the refolding of a specific protein and in many cases seems to have been overlooked. (e.g. Rudolph *et al.* (1990)).

4.8 The effect of protein concentration on the aggregation of lysozyme.

Aggregation was studied at different concentrations of denatured protein in a Hi-tech Scientific stop-flow spectrophotometer. Equal volumes of denatured reduced protein in 0.1M acetic acid and refolding buffer (0.2 M Tris HCl, pH 8.2, 3mM oxidised glutathione, 0.3mM reduced glutathione) were mixed rapidly and the change in turbidity of the solution measured over time at 450nm. The initial concentration of protein in acetic acid was altered to give the correct concentration after mixing. Figure 4-14 shows the results of the effect of protein concentration on aggregation.

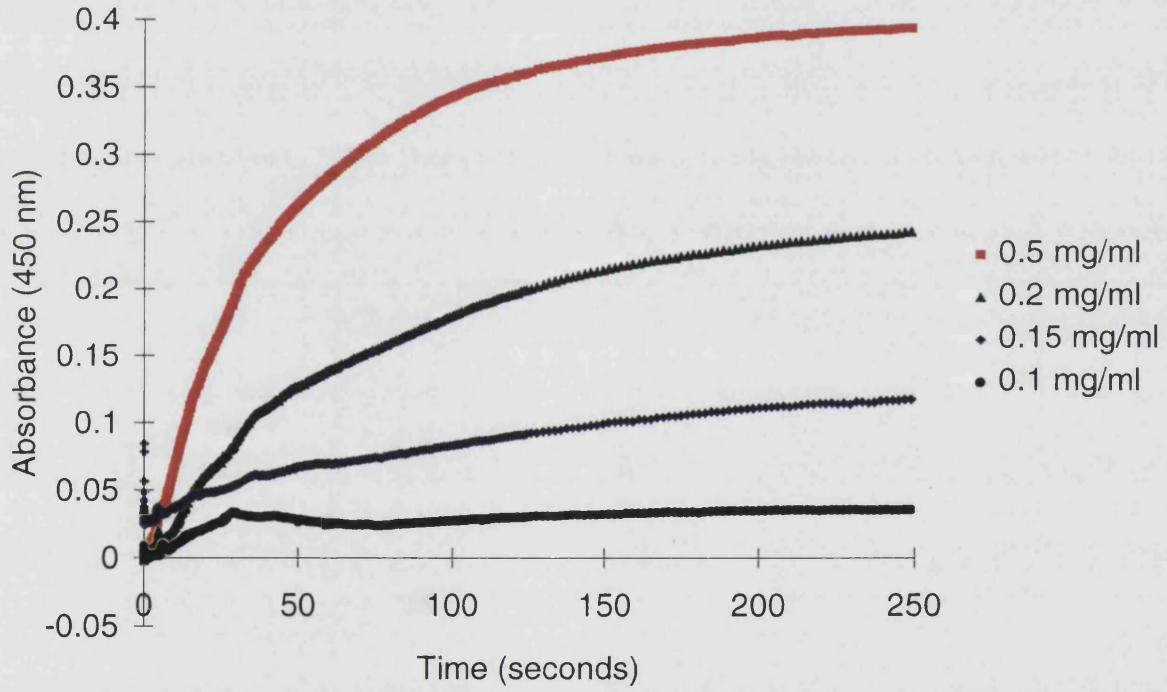

Figure 4-14 The effect of protein concentration on the aggregation of lysozyme

As can be seen from Figure 4-14, as the concentration of protein in the refolding buffer is increased the rate and extent of aggregation increases. The relationship between protein concentration and the rate of aggregation can be described by second order reaction kinetics. (See Section 5.2)

The results are similar to those obtained by Zettlemeissel *et al.* (1979) for the aggregation of lactic dehydrogenase (LDH) and to those obtained by Plomer and Gafni (1993) for the aggregation of glucose-6-phosphate dehydrogenase (G6PD). In both cases they reported that as the protein concentration is increased the rate and extent of aggregation increases. De Young *et al.* (1993) have performed an extensive study of the aggregation of apomyoglobin. Apomyoglobin is a small protein, 17,000 Daltons, with no di-sulphide bridges. De Young *et al.* (1993) showed that aggregation increased significantly with an increase in protein concentration. In this study the aggregation reaction occurred over minutes, like G6PD, as opposed to seconds in the cases of lysozyme, LDH. This is thought to be due to the fact that these proteins have no di-sulphide bonds and when the denaturant is diluted that the protein rapidly forms a much more stable intermediate than di-sulphide bonded proteins and only specific domains are available as sites for aggregation. This idea has been hypothesised previously. (Mitraki and King (1989))

De Young *et al.* (1993) observed a lag before a change in turbidity of the solution being studied was detected. This is taken by some to indicate that protein aggregation occurs by a nucleation and growth phenomenon. However it is more likely that the time lag is a result of the fact that at low concentrations of aggregates not enough light is scattered to be detected by turbidity measurements. In this case more sensitive methods of analysis, such as fluorimetry, could be used.

Turbidity is not necessarily the best method for studying aggregation if an in depth understanding of the physico-chemical interactions which lead to aggregation are to be fully understood. This is because aggregates have to grow to a significant size before they are detected by turbidity measurements. However, these studies are significant enough to allow an approximation of the rate of reaction with respect to concentration.

5. Modelling of the Refolding and Aggregation of Lysozyme

In this Chapter reaction mechanisms for both the refolding and aggregation of lysozyme are proposed. The rate constants for each of the reactions are calculated from experimental data. By modelling these two reactions simultaneously a competitive model is formed. The results predicted by the model are then compared to the experimental results. By introducing a selectivity parameter ideal reactor conditions are predicted. Using the model, different refolding strategies are investigated. In this case the step-wise and continuous addition of denatured lysozyme to the refolding buffer are evaluated. The results of the model are then compared with experimental results.

It should be noted that this particular model applies specifically to monomeric proteins. For the modelling of oligomeric proteins one or more equations would need to be added to describe the association of the protein sub-units. The same methodology can still however be applied. Rate constants for the association of protein subunits have been calculated. The order of reaction has been investigated and has been shown to be second order for the association of porcine mitochondrial malate dehydrogenase (m-MDH) sub-units. The rate of reaction was found to be 3.1×10^{-4} $M^{-1}s^{-1}$ (Jaenicke (1986)). Had Jaenicke investigated the aggregation of m-MDH he could have applied this type of model in order to optimise the refolding process.

5.1 Kinetics of refolding

By measuring the yield of active lysozyme over time for the refolding process all the rate limiting steps can be combined into one reaction. Assuming first order rate kinetics (Thatcher and Hitchcock (1994)) the refolding of lysozyme can be described by the following equation.

$$\frac{dC_N}{dt} = k_1 C_D \qquad \text{Equation 5-1}$$

where C_N is the concentration of native protein (mg/ml), C_D is the concentration of denatured protein (mg/ml), k_1 is the first order rate constant (min^{-1}) and t is time (min).

given

$$C_O - C_N = C_D \qquad \text{Equation 5-2}$$

C_O = initial concentration of protein (mg/ml)

we can substitute to give Equation 5-3 where the only unknown is k_1.

$$\ln\left(\frac{C_O - C_N}{C_O}\right) = k_1 t \qquad \text{Equation 5-3}$$

This equation assumes no other reaction is taking place. As the refolding yield obtained is less than 100% it is obvious that some reaction other than refolding is taking place. This assumption is necessary to obtain an estimate to the first order refolding rate constant. The approach is valid at low concentrations of denatured protein as aggregation is minimal. Plotting $\ln((C_O-C_N)/C_O)$ against t for the data in Figure 4-9 gives $-k_1$ as the gradient of the line through the data (Figure 5-1).

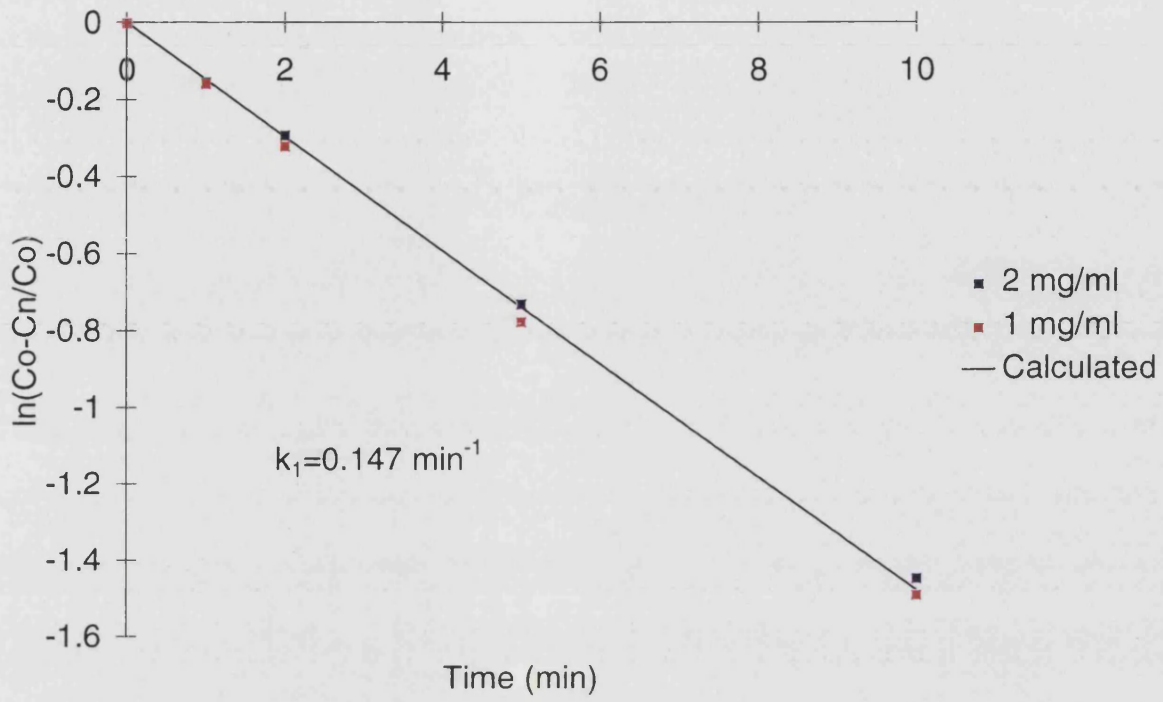

Figure 5-1 Calculation of k_1, the first order rate constant for the refolding of lysozyme.

This approach is applied to the results obtained for the refolding from both 1mg/ml and 2mg/ml denatured reduced protein in 0.01M acetic acid to give a final concentration of 0.01 mg/ml. As 95% conversion was achieved in both cases the initial concentration C_o is taken as 0.0095 mg/ml.

From this data k_1 is calculated to be 0.147 min^{-1}. This is an order of magnitude higher than that calculated for the refolding of lactic dehydrogenase (0.01 s^{-1}, Zettlemeissel *et al.* (1979)). The rate at which a protein regains its native structure seems to be a function of the size and complexity of the protein being refolded. As the protein becomes larger and more complex the rate of refolding is generally reduced. Tissue plasminogen activator which has 17 di-sulphide bonds takes several hours to refold (Rudolph *et al.* (1990)).

The kinetics of refolding for a number of different proteins have been followed by several different techniques and on several different time-scales. For a review of these techniques see the Appendix: Section 11.2. Many of these techniques are used for conformational studies which are attempting to elucidate folding pathways and expand our knowledge of why and how proteins fold. In many cases the protein is not reduced during these experiments and activity is not measured. These studies have shown that for the refolding of lysozyme that alpha-helices form very rapidly and that the conformation of the folded structure after a few milliseconds resembles quite closely the structure of the native protein. It is suggested that the stabilisation of these alpha helices is the rate limiting step in achieving the active protein (Chaffotte *et al.* (1992)).

It is important to note that in some of these experiments the protein has not been fully reduced. With the di-sulphide bridges intact the number of possible conformations the protein can assume whilst refolding is greatly reduced and although these experiments provide an insight into the time scale at which conformational changes occur, until similar experiments are reproduced using reduced protein the implications of the data must be interpreted very carefully.

5.2 Kinetics of Aggregation

The general rate equation for a reaction with a single reactant in a constant volume batch reactor

$$\frac{dC_Y}{dt} = k(C_X)^{\psi} \qquad \text{Equation 5-4}$$

where C_Y is the concentration of product (mg/ml), C_X is the concentration reactant (mg/ml), k is the rate constant and t is time (min) and ψ is the order of reaction.

If the perikinetic growth of aggregates is assumed aggregation can be described as a second order process (Equation 5-5). The rate constant in this case is assumed to be determined by the diffusivity and diameter of the denatured protein. This would be accurate for the aggregation of monomers where convective forces are not important. This is obviously not the case in the aggregation of refolding proteins where large multimers are formed quickly. However, as a first approximation, aggregation can be described by two molecules reacting to form a larger molecule. This is essentially a second order process. The rate of reaction for different sized multimers may be different but the order of reaction is still second order. It is not unreasonable therefore to approximate the aggregation reaction to a single second order process with a single apparent rate constant . Also, the model fits well to experimental data (Zettlemeissel *et al.* (1979)) and is valid for the refolding conditions used.

$$\frac{dC_A}{dt} = k_2(C_D)^2 \qquad \text{Equation 5-5}$$

where C_A is the concentration of aggregated protein (mg/ml), C_D is the concentration of denatured protein (mg/ml), k_2 is the second order rate constant (mg/mg min) and t is time (min)

At 0.5 mg/ml only minimal reactivation of the protein is achieved. Therefore it is reasonable to assume that aggregation is the only reaction taking place. The concentration of denatured and aggregated protein at any given time is described in Equation 5-6.

$$C_O - C_A = C_D \qquad \text{Equation 5-6}$$

Substitute Equation 5-6 into Equation 5-5:

$$\frac{1}{C_O - C_A} = -k_2 t \qquad \text{Equation 5-7}$$

Applying this to the data for aggregation at 0.5 mg/ml the following graph is obtained. We assume that all the protein added takes part in the reaction (i.e. C_0=0.5 mg/ml and at t=∞ C_A=0.5 mg/ml).

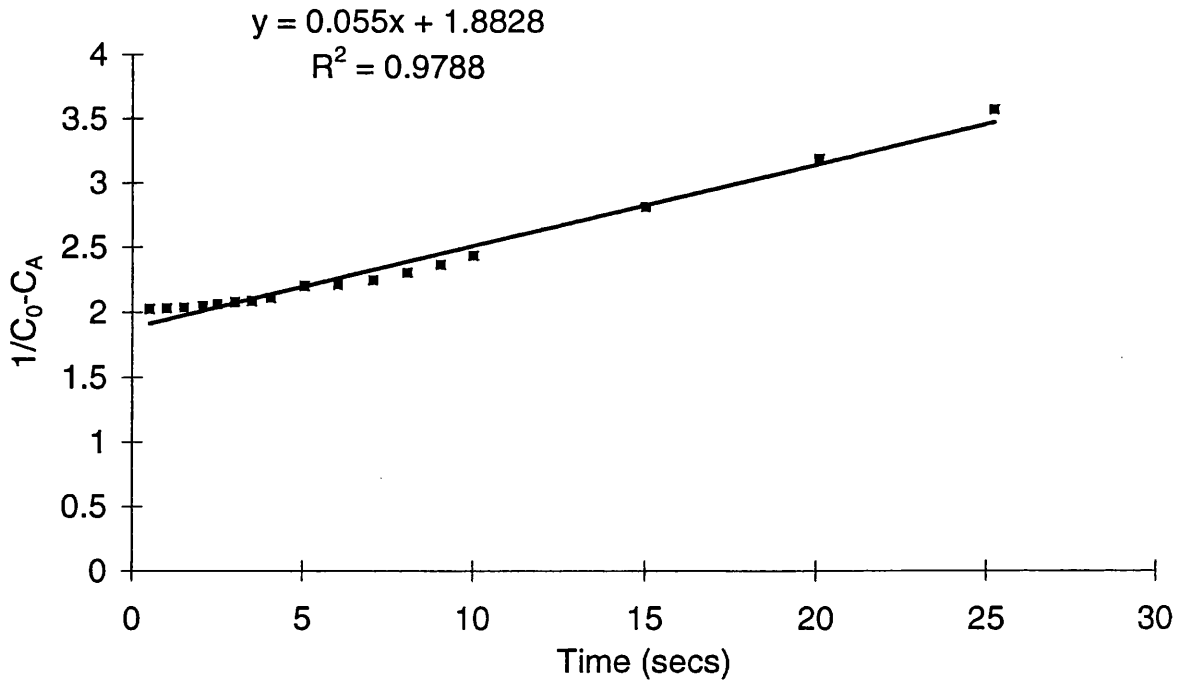

Figure 5-2 Calculation of the second order rate constant for aggregation

Using the equation above the following results were achieved for values of C_{DO} ranging from 0.015 mg/ml to 0.2 mg/ml.

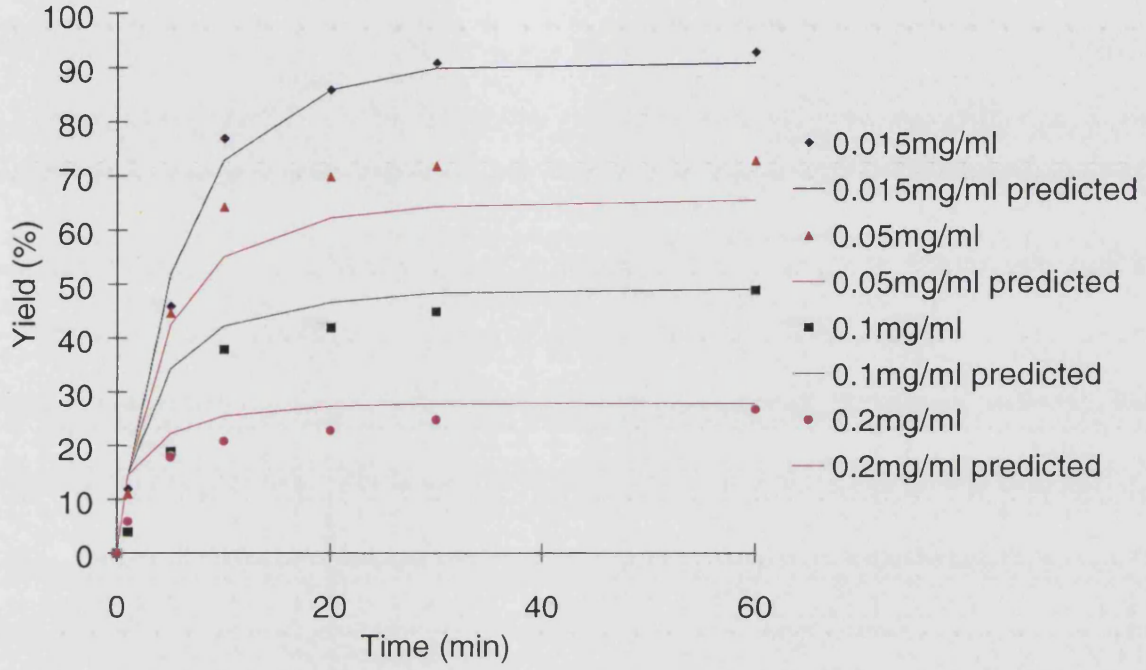

Figure 5-3 Experimental data and model for the refolding of lysozyme at different protein concentrations.

The model fits the experimental data well. At 0.2 mg/ml and 0.1 mg/ml the model overpredicts the rate of refolding but the final predicted yield is within 1 % of the experimental results.

It has been shown that it is possible to approximate the refolding of reduced denatured lysozyme to a simple two state competitive model. The model is based upon a first order reaction for refolding and a second order reaction for aggregation. The rate constants for both reactions were calculated by performing experiments at different concentrations of substrate and following the reaction over a suitable time-scale. The rate constant for refolding was calculated to be 0.147 min^{-1} and the rate constant for aggregation was calculated to be 3.3 ml/mg min.. This model can be used to predict optimum

The calculated rate constant assuming second order kinetics is 0.0558 ml/mg s (0.78 $\mu M^{-1}s^{-1}$). Zettlemeissel *et al.* (1979) calculated the apparent order of reaction for the aggregation of lactic dehydrogenase (LDH) to be 2.5 and the apparent rate constant to be 3.9 $\mu M^{-1}s^{-1}$. Plomer and Gafni (1993) calculated the rate constant for the aggregation of glucose-6-phosphate dehydrogenase (G6PD) to be 2.19 $\mu M^{-1}min^{-1}$. The rate constant ofr the aggregation of lysozyme is lower than the rate constants calculated for LDH and G6PD. This is thought to be due to the fact that lysozyme is considerably smaller than LDH and G6PD. When the denaturant is diluted the protein rapidly forms a more stable intermediate and less sites are available for aggregation (Mitraki and King (1991).

Using the calculated values of the rate constants for refolding and aggregation a simple iterative model was constructed using a spreadsheet. (Excel, Microsoft)

$$\frac{dC_N}{dt} = 0.147C_D \qquad \text{Equation 5-8}$$

$$\frac{dC_A}{dt} = 3.3(C_D)^2 \qquad \text{Equation 5-9}$$

By combining these equations and integrating the following expression for yield of native lysozyme with respect to time and protein concentration can be formed.

$$y_N = \frac{C_N}{C_{D0}} = \frac{0.147}{3.3C_{D0}} \ln\left(1 + \frac{3.3C_{D0}}{0.147}\left(1 - e^{-0.147t}\right)\right) \qquad \text{Equation 5-10}$$

where C_{D0} is the initial concentration of denatured protein in the refolding buffer (mg/ml)

refolding processes. At 0.05 mg/ml the model underpredicts the refolding yield. At 0.015 mg/ml the model is most accurate predicting the rate and final yield of refolding to within 5% at any time. The results show that describing refolding by a first order reaction is accurate. The fact that the predicted results at 0.05 mg/ml are not as accurate as at higher concentrations suggests that the aggregation process is more complicated than the second order reaction used to describe it. The second order rate constant is based on data taken at relatively high protein concentrations (greater than 0.5 mg/ml). The aggregation reaction at intermediate concentrations obviously does not behave in the same manner as at high concentrations. Further work will need to be performed if the model presented here is to be improved.

It has been shown that lysozyme refolding can be described as a first order reaction. The aggregation of lysozyme has been followed at different concentrations of lysozyme and can be described as a second order process. The apparent rate constant for refolding of lysozyme was found to be 0.147 min^{-1} and the apparent rate constant for aggregation was found to be 3.3 $mgml^{-1}min^{-1}$. Using these rate constants, a competitive model of refolding versus aggregation has been written. The experimental results agree well with the results predicted by the model

5.3 Process options for maximising the yield of refolded lysozyme

Although experimental results suggest that the concentration of denatured protein should be kept low to obtain high process yields this has never been described in terms of selectivity, S. The selectivity parameter is defined as the rate of production of desired product over the rate of production of undesired products. For two competing reactions of order α_1 and α_2 and rate constants of k_1 and k_2 respectively the selectivity, S, can be described by the following equation.

$$S = \frac{r_1}{r_2} = \frac{k_1}{k_2} C_D^{\psi_1 - \psi_2} \qquad \text{Equation 5-11}$$

where r_1 is the rate of the desired reaction, r_2 is the rate of the undesired reaction, k_1 is the rate constant for reaction 1, k_2 is the rate constant for reaction 2, ψ_1 is the order of reaction 1 and ψ_2 is the order of reaction 2.

Experimental results have shown that protein refolding yields are highest at low concentrations of refolding protein. This is due to a competitive aggregation reaction. This can be described by the mass transfer processes involved. As the refolding protein becomes more dilute the number of interactions between refolding molecules is reduced. This in turn allows the protein to refold. Selectivity describes this physical process in terms of the apparent rate constants and orders of reaction of the refolding and aggregation reactions. The selectivity does not take into account the physical processes involved but it can be used as a tool to identify optimum conditions for maximising the yield of refolded protein

Let $\psi_2 - \psi_1 = a$. For the refolding of lysozyme a = 1.

$$S = \frac{r_R}{r_A} = \frac{k_1}{k_2 C_D^{\psi_A - \psi_R}} = \frac{k_1}{k_2 C_D^{\,a}} \qquad \text{Equation 5-12}$$

where r_R is the rate of refolding, r_A is the rate of the aggregation, k_1 is the rate constant for refolding, k_2 is the rate constant for aggregation, ψ_1 is the order of refolding and ψ_2 is the order of aggregation.

It can be seen from Equation 5-12 that for S to be as high as possible C_D should be as low as possible. As the concentration of denatured protein should be kept low at all times a continuously stirred reactor (CSTR) should be used rather than a batch or plug flow reactor (PFR). This allows a steady build up of product in the reactor whilst minimising the production of undesired product. This is the rational basis for continuous and batch-fed refolding.

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5.4 Modelling process options to maximise the refolding yield of lysozyme.

The analysis of the selectivity shows that C_D should be kept low at all times. However, although this gives a high yield it also means that the product concentration is low leading to high process volumes. C_D can be kept low by adding some denatured reduced lysozyme to refolding buffer, allowing it to refold and then adding some more denatured material and allowing it to refold. By doing this the selectivity will be kept high and the concentration of refolded lysozyme can be increased whilst maintaining a high overall yield. Using the model described earlier the following results are obtained.

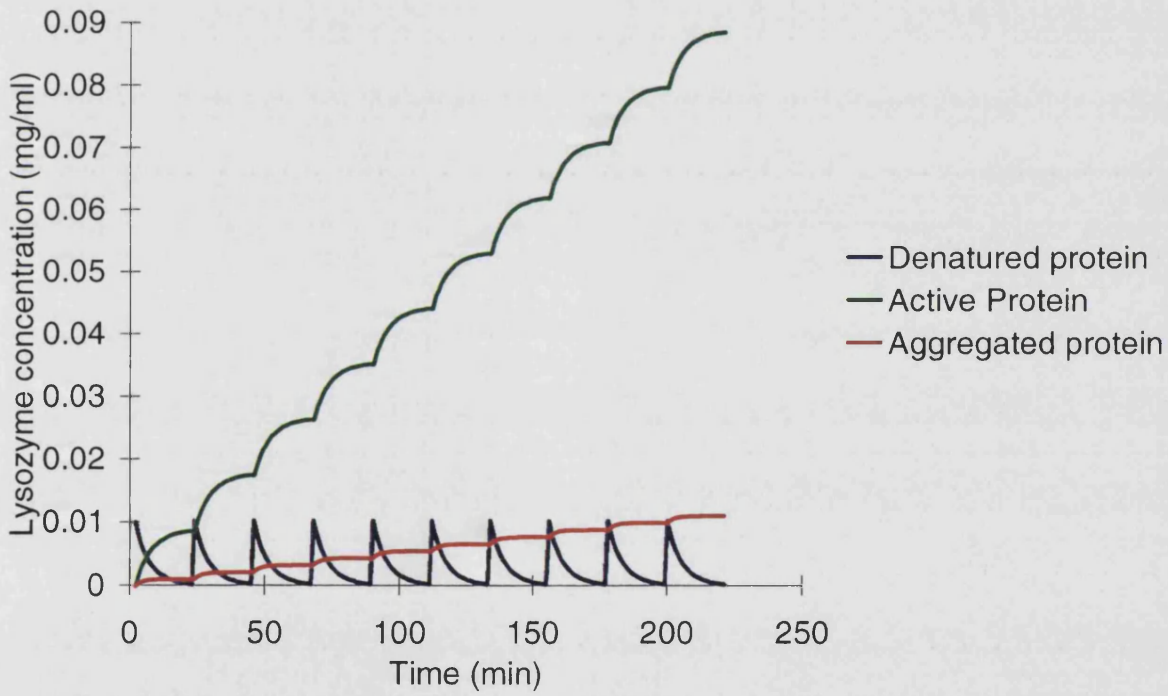

Figure 5-4 Predicted performance of step-wise addition of reduced denatured lysozyme.

Figure 5-4 shows that by performing 10 additions of 0.01 mg/ml lysozyme into the same refolding buffer that lysozyme can be efficiently refolded to 0.1mg/ml. A step length between additions of twenty minutes was chosen as

this is the rime for the refolding reaction to reach 99% completion. The overall predicted yield for the fed-batch process is 88% as opposed to 47% for a single step-addition. Although this is a significant increase in the overall yield of the refolded protein the time taken to refold the protein is significantly longer in the fed-batch process, nearly three hours as opposed to 20 minutes for the single step-addition. The reason for the difference is well illustrated in Figure 5-4. For nearly half the reaction time the concentration of denatured protein is practically zero. This represents a large process inefficiency.

5.4.1 Fed-Batch Addition of Denatured Reduced Lysozyme. Comparison of the predicted results with experimental data.

Fed batch refolding is essentially cycles of batch refolding performed in the same refolding buffer. From model predictions (See Section 5.4) this should result in a gradual increase in the protein concentration in the refolding buffer whilst minimising aggregation.

Renaturation was initiated by adding 100 µl of a 20 mg/ml solution of denatured reduced lysozyme in 6M GuHCl 0.15M DTT to 200 ml of renaturation buffer to give a solution of 0.01mg/ml of lysozyme (0.1M Tris-HCl, pH8.2, 1 mM EDTA, 3mM reduced glutathione and 0.3 mM oxidised glutathione) and the subsequent rise in specific activity of the refolding solution was monitored using the activity assay. After a given time another 100 µl of a 20 mg/ml solution of denatured reduced lysozyme was added to the refolding buffer. This process was then repeated 6-10 times depending on the specific experiment. The time allowed between consecutive additions was 10, 20 and 30minutes. Aggregation was followed at 450 nm. All experiments were performed at 25°C

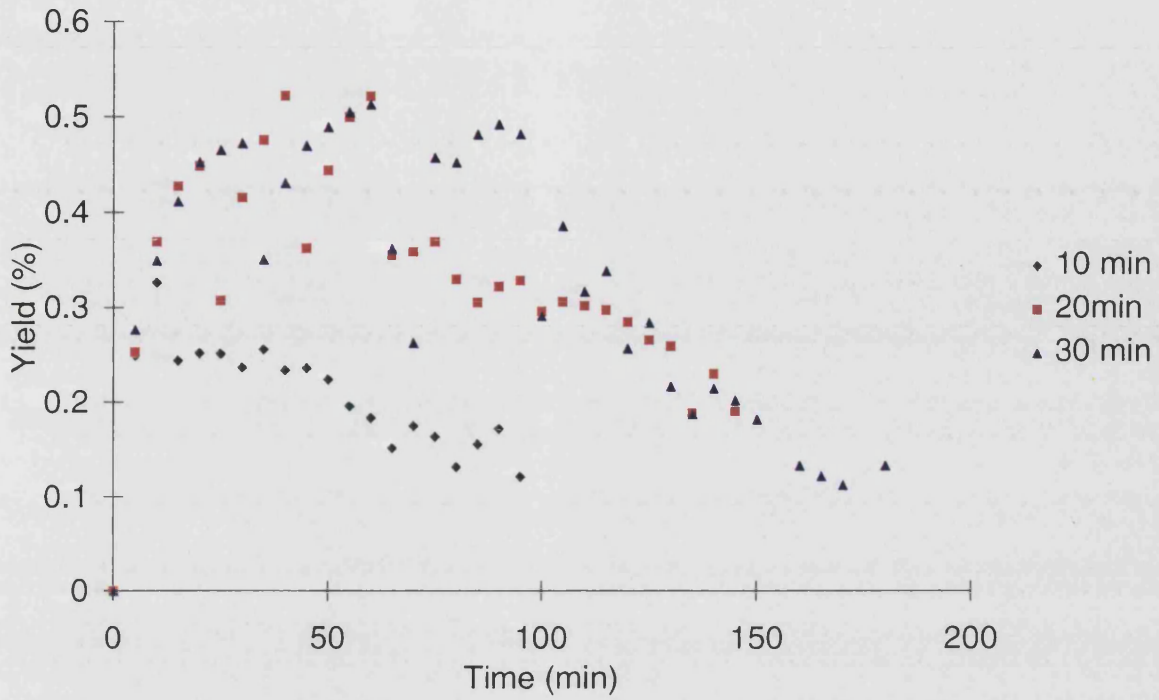

Figure 5-5 The effect of rate of addition of denatured lysozyme in 6M GuHCl 0.15M DTT on the yield of native lysozyme in the step-wise addition of lysozyme.

For the step-wise addition of lysozyme with a step length between additions of 10 minutes a very low yield is obtained. For the first addition the yield increases to 34% as expected. When more protein is added after ten minutes the yield drops to 25%. The yield then decreases over time as more protein is added. The final yield after 10 additions is 12%. As the time between additions is increased from 10 to 20 and 30 minutes there is little difference in the observed results. In both cases the refolding yield follows the profile of three consecutive batch refolding experiments. After the fourth addition of protein there is a sudden decrease in yield which continues after the fifth addition.

The results for additions every ten minutes show a very low yield being obtained. This may be due to the fact that after ten minutes the intermediates which proliferate in the refolding buffer are not stable. These intermediates interact with the newly added denatured material causing the

low yield. Goldberg *et al.* (1991) performed an experiment which was essentially two additions. They varied the time between additions and found that after 7.5 minutes the folding intermediates from the first addition were stable and did not interact with newly added denatured reduced lysozyme. The results in Figure 5-5 suggest that for these experiments that the corresponding time taken for the refolding lysozyme to reach a stable intermediate lies between 10 and 20 minutes. The discrepancy may be due to the difference in the initial conditions of the denatured protein prior to refolding. Goldberg *et al.* (1991) refolded lysozyme which had been denatured in GuHCl and then dialysed against acetic acid. Reduced denatured lysozyme after dialysis has been shown to have a more compact structure than reduced denatured lysozyme in 6M GuHCl 0.15M DTT Saxena and Wetlaufer (1970). This more compact denatured protein would logically take less time to reach a stable intermediate state which does not interact with newly added denatured material. This is exemplified by the fact that higher refolding yields can be achieved by refolding from denatured lysozyme in acetic acid than refolding from denatured lysozyme in GuHCl. In addition to this, the increase in concentration of denaturant and reducing agent is at a critical level after only forty minutes. This is illustrated by the rapid decrease in yield after that time.

The results for the 20 and 30 minute addition times follow the predicted results for the first three additions. The sudden drop in yield and deviation from the model prediction after the fourth addition of lysozyme is probably due to the increasing concentration of denaturant and reducing agent in the refolding buffer. After each addition of denatured reduced lysozyme the conditions in the refolding buffer become less favourable for refolding. After the fourth addition of denatured protein, the concentration of GuHCl in the refolding buffer is 0.024 M and the concentration of DTT is 0.6 mM. According to the batch experiments carried out (See Section 4.3 and Section 4.4), under these conditions practically zero yield is achieved. The continued

decrease over time may be due to refolded protein being inactivated either by the concentration denaturant and reducing agent or by unfavourable interactions with aggregating protein.

Again the effect of residual denaturant and reducing agent concentration have been shown to be limiting in refolding reduced denatured lysozyme. In order to try to keep the solvent conditions constant step-wise addition of protein was repeated using denatured reduced lysozyme in acetic acid. Refolding yield has been shown to be relatively insensitive to changes in pH over the range 8-9. (See Section 4.2). Therefore the change in solvent conditions due to the step-wise addition of 0.1M acetic acid should have a negligible effect on the refolding yield.

In order to investigate the step-wise addition of lysozyme without the detrimental effects of GuHCl and DTT being present the following experiment was performed. Renaturation was initiated by adding 2 ml of a 1 mg/ml solution of denatured reduced lysozyme in 0.1 M acetic acid to 200 ml of renaturation buffer (0.1M Tris-HCl, pH8.2, 1 mM EDTA, 3mM reduced glutathione and 0.3 mM oxidised glutathione) to give a solution of 0.01mg/ml of lysozyme and the subsequent rise in specific activity of the refolding solution was monitored using the activity assay. After a 20 minutes another 2 ml of a 1 mg/ml solution of denatured reduced lysozyme was added to the refolding buffer. This process was then repeated 6 times. Aggregation was followed at 450 nm. All experiments were performed at 25°C.

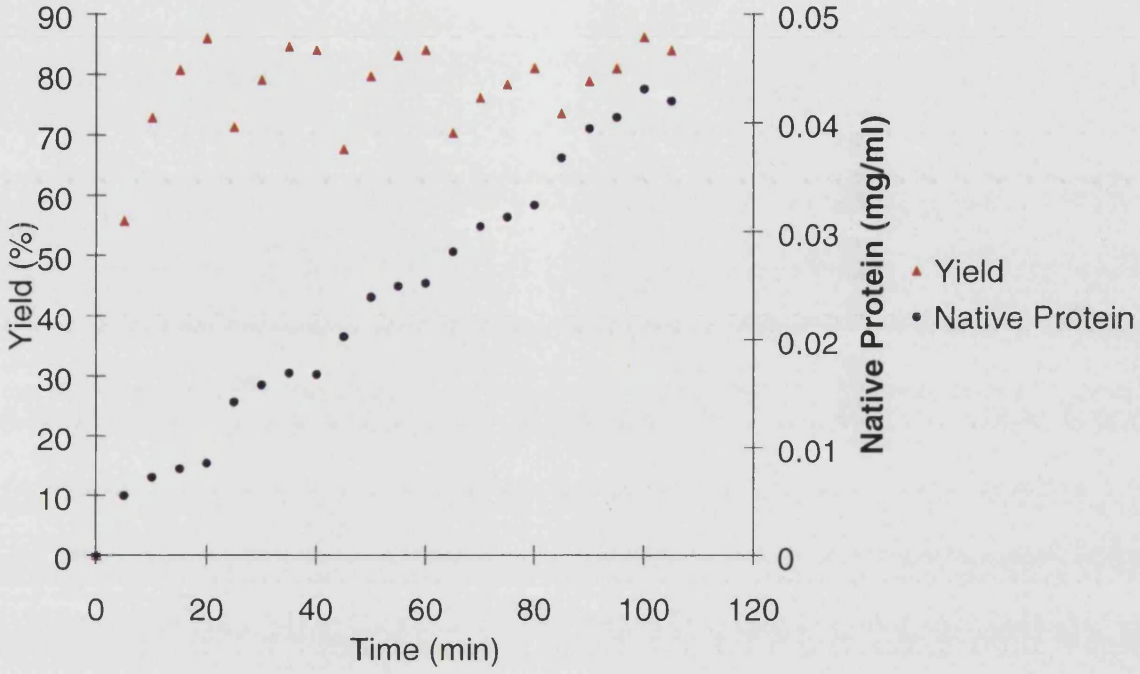

Figure 5-6 The step-wise addition of lysozyme denatured in 0.1M acetic acid.

From the results in Figure 5-6 it can be seen that the step-wise addition of denatured reduced lysozyme in 0.1 acetic acid to the refolding buffer yields results similar to those predicted by the model (See Section 5.3). The overall yield was 84% compared to 52% for the single step batch process.

Rudolph *et al.* (1990) are the only group to have published significant data on the step-wise addition of refolding proteins. This publication is in the form of a patent and does not identify the problems associated with using this method when refolding from denatured material which has not been dialysed against acetic acid. The study involved the refolding of lysozyme, lactic dehydrogenase and recombinant tissue plasminogen activator. In each case it was found that pulse reactivation increased the yield of refolding over a single batch addition. It was also shown for each of the proteins that there exists an optimal time between additions. Rudolph *et al.* (1990) demonstrated that the

optimal time between additions for the refolding of lysozyme lies between 10 and 20 minutes. This is similar to the results described in Section 5.4.1.

For the refolding of lysozyme the size of additions is higher than the additions used in this study. The lowest addition was 0.04 mg/ml. Four times higher than that used in this study. Using a step length of 20 minutes, after 24 hours and 20 pulses they only achieved a refolding yield of 17%. As opposed to 2.3% for the single step addition. This yield is still lower than that expected from our results. The only explanation that can be given for this is that Rudolph *et al.* (1990) like Goldberg *et al.* (1991) dialysed lysozyme against 0.1M HCl as opposed to acetic acid. When Goldberg refolded lysozyme under similar conditions to those used in Section 4.6 they obtained a lower yield than that described in Section 4.6. Work by Saxena and Wetlaufer (1970) supports the results in Section 4.6.

Tereshima *et al.* (1996) investigated the use of a plug-flow reactor. The idea being to minimise aggregation by reducing axial mixing using a packed column. As part of their justification for the use of this type of reactor they performed fed-batch experiments. They showed that fed-batch gave no improvement over a single batch addition. The reason for this is that the efficiency of each of the steps was approximately the same as the single addition. For a fed-batch system to improve the refolding process the efficiency/yield of each step must be significantly higher than the efficiency of the single addition.

It has been shown in this chapter that step-wise addition of reduced denatured lysozyme in 0.1M acetic acid can be used to refold lysozyme to 0.1 mg/ml with a significantly improved yield over a single batch addition. It has also been shown that these results agree well with the predicted results in Section 5.4. Refolding of reduced denatured lysozyme in 6M GuHCl and 0.15M DTT does not follow the predicted model well. This is thought to be due to the changes in the chaotropic nature and redox potential of the system

as each sample of reduced denatured lysozyme is added. Whether or not batch refolding will be economically viable will depend on the trade off between the improved yield and the increased residence time in the reactor.

5.4.2 Continuous Refolding of Reduced Denatured Lysozyme

In Section 5.4.1 it was shown that if the refolding conditions are kept constant (i.e. that the protein is refolded from protein in 0.1 M acetic acid) that efficient refolding at high concentration can be achieved by step-wise addition of lysozyme. In Section 5.4.1 it was shown that batch refolding was sub-optimal because the concentration of denatured lysozyme (reactant) was low for approximately 50% of the reaction time. It was suggested that the reaction time could be reduced by adding the protein to the system continuously. By doing this the concentration of protein being added can be kept at the critical concentration. That is the maximum concentration of reduced denatured protein in the refolding buffer at which the refolding reaction still predominates. The rate of addition must equal the rate of refolding. This can be modelled using the same two state competitive model used for the fed-batch system and assuming that the concentration of denatured protein is constant.

$$y_N = \frac{C_N}{C_D} = \frac{0.147}{3.3C_D}\ln\left(1+\frac{3.3C_D}{0.147}\left(1-e^{-0.147t}\right)\right) \qquad \textbf{Equation 5-13}$$

where C_D is the concentration of denatured protein in the refolding buffer (mg/ml)

By assuming that the concentration of denatured lysozyme in the refolding buffer is constant, the rate of change of the native lysozyme concentration and yield are constant. This assumption is valid if the rate of addition of denatured protein equals the rate of refolding and aggregation. This model also assumes that the refolding conditions do not change significantly and do not affect the rate constants of refolding and aggregation.

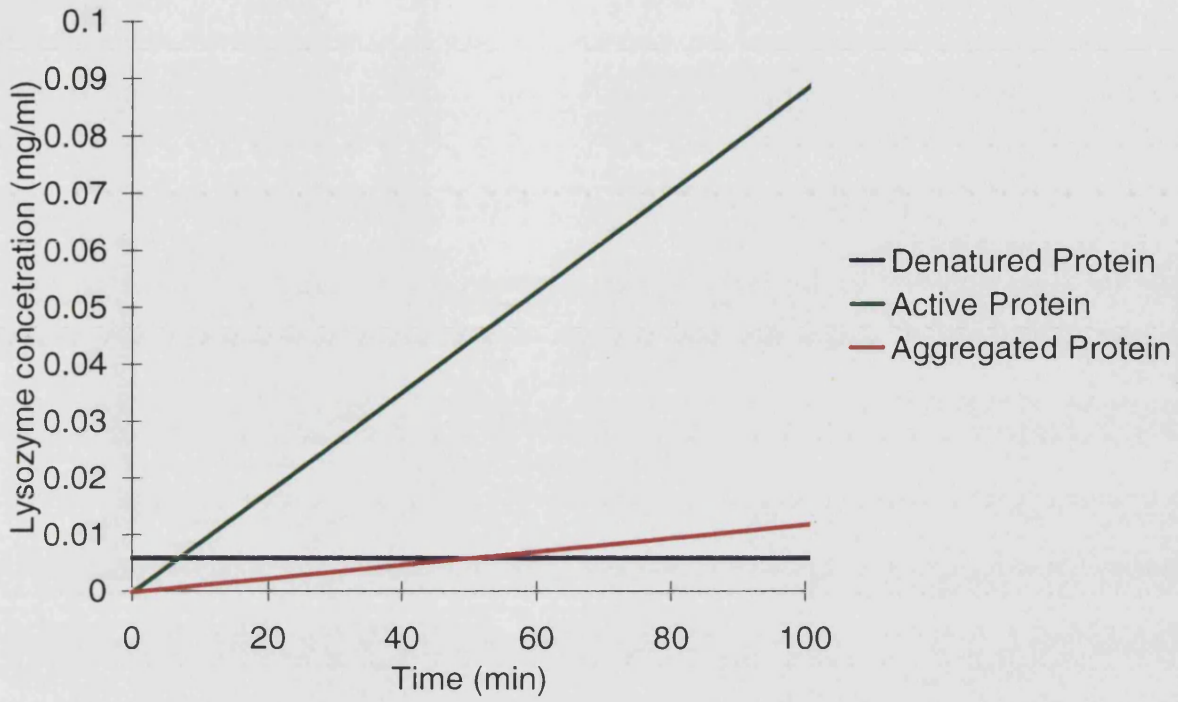

Figure 5-7 Continuous addition of reduced denatured lysozyme to the refolding buffer increases the process yield whilst maintaining a high protein concentration and minimising process time.

Figure 5-7 shows that by keeping the concentration of reduced denatured lysozyme in the refolding buffer at 0.006 mg/ml a concentration of active protein of 0.088 mg/ml can theoretically be achieved after 1 hour and forty minutes. This is equivalent to an addition rate of 0.001 mg/ml min or 0.2 mg/min (refolding buffer volume = 200 ml). The process yield is high, 88 %, almost double the single addition of 0.1 mg/ml. The process time is significantly lower than step-wise addition and only five times higher than for the single step addition.

The model shows quite clearly the advantage of adding denatured protein continuously. By using this approach an existing single addition process can be dramatically improved with minimum expenditure. The only drawback of the method is that to achieve a high yield the length of time involved in

producing the product is increased. Whether or not the increase in yield justifies the extended reaction time requires an economic assessment.

5.4.3 Continuous Addition of Denatured Reduced Lysozyme. Comparison of the predicted results with experimental data.

A 0.1 mg/ml solution of denatured reduced lysozyme in 0.1M acetic acid was added to 200 ml of 0.1M Tris-HCl, pH8.2, 1 mM EDTA, 3mM reduced glutathione and 0.3 mM oxidised glutathione using a Watson-Marlow 1010 peristaltic pump (Watson-Marlow Ltd., Cornwall). To achieve different rates of addition of denatured protein to the system the concentration of denatured lysozyme (0.1 mg/ml) was kept constant and the flow-rate was varied. As the flow-rates used were low the feed pipe from the pump was kept immersed in the refolding buffer at all times to avoid droplet formation. Refolding was monitored using the activity assay. Aggregation was monitored at 450 nm. The system was gently stirred using a magnetic stirrer and all experiments were performed at 25°C

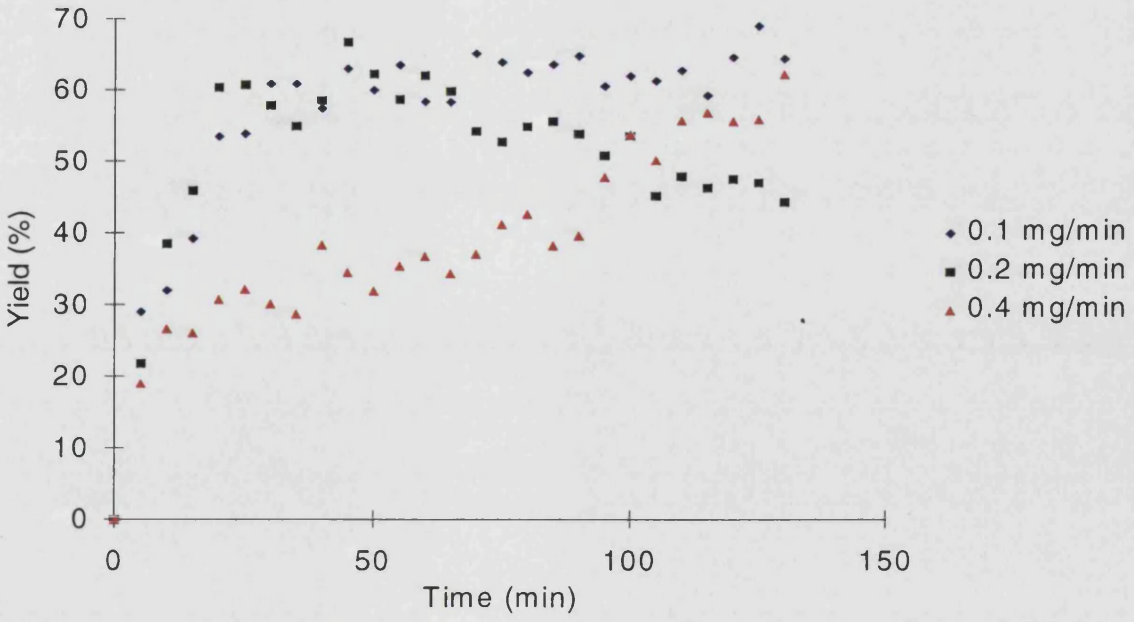

Figure 5-8 The effect of rate of addition of lysozyme on the yield of native lysozyme in the continuous refolding of lysozyme.

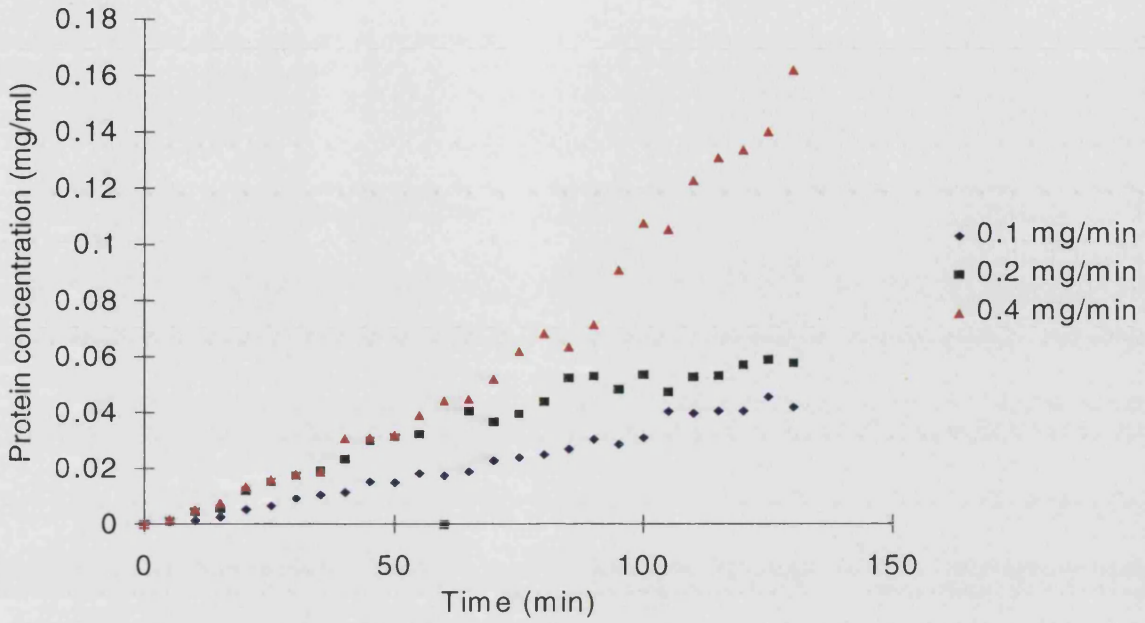

Figure 5-9 The effect of rate of addition of lysozyme on the yield of native lysozyme in the continuous refolding of lysozyme

The results of the effect of the rate of addition on the yield and concentration of refolded lysozyme are shown in Figure 5-8 and Figure 5-9. At an addition rate of 0.1 mg/min the yield increases to approximately 60% and remains constant, concomitantly the concentration of refolded lysozyme increases linearly. At an addition rate of 0.2 mg/min the yield increases to approximately 60% and then slowly decreases over time. This results in the concentration of native lysozyme increasing linearly with time and then rate of increases in the concentration slowly decreases. For an addition rate of 0.4 mg/min the yield increases almost linearly from 20% to 60% over time. This results in an exponential increase in the concentration of native lysozyme.

The 0.1 mg/min addition follows the predicted model well. The yield remains constant and the concentration of refolded protein increases linearly with time. With the addition rate of 0.2 mg/min the initial increase in yield is as expected. The decrease in yield is probably due to the fact that the rate of addition exceeds the rate of refolding. The concentration of denatured protein

in the system exceeds the critical value for aggregation and causes the decrease in yield. This is supported by the fact that the aggregation measured at 450nm increases rapidly after 50 minutes

The increase in yield with respect to time for the 0.4 mg/min addition rate is difficult to explain. The rate of aggregation over time increases initially and then seems to stop. It may be the case that as the concentration of refolded protein in the system increases the stability of the refolding intermediates increases. The aggregation reaction may be inhibited by the increase of native molecules in the refolding system. These molecules would have a similar effect to PEG (Cleland and Wang (1990)) in the system and may well prevent aggregation by limiting the mass transfer of refolding intermediates prone to aggregation.

5.5 Conclusions

Lysozyme refolding can be described as a first order reaction. The aggregation of lysozyme has been followed at different concentrations of lysozyme and can be described as a second order process. The apparent rate constant for refolding of lysozyme was found to be 0.147 min^{-1} and the apparent rate constant for aggregation was found to be 3.3 $mgml^{-1}min^{-1}$

Using these rate constants, a competitive model of refolding versus aggregation has been written. The experimental results agree well with the results predicted by the model. A selectivity term based on the two competitive reactions has been introduced. It has been shown that refolding yields can theoretically be enhanced by stepwise or continuous addition of denatured lysozyme. Denatured reduced lysozyme in 0.1M acetic acid follows the predicted results well whereas denatured reduced lysozyme in 6M GuHCl and 0.15M DTT does not. This has been shown to be due to the increasing concentrations of both guanidine hydrogen chloride and dithiothreitol in the refolding buffer. Continuous addition of denatured protein to refolding buffer

is likely to be effective for improving the refolding yield of any given protein regardless of the rate of refolding and the rate of aggregation.

6. Economic Evaluation of Protein Refolding

From the experimental observations and theoretical predictions presented earlier it is evident that many factors affect the refolding yield of lysozyme. In this Chapter the influence of changes in the refolding strategy on the process cost is explored. Much work to date has been aimed at understanding refolding. Little attention has been paid to the relationship between yield and the concentration of active protein with respect to process economics. The final concentration of active protein in the refolding buffer is as important as the yield. Figure 6-1 shows that for the refolding of lysozyme although the yield decreases as the amount of protein added to the refolding buffer is increased, the concentration of active protein in the solution increases.

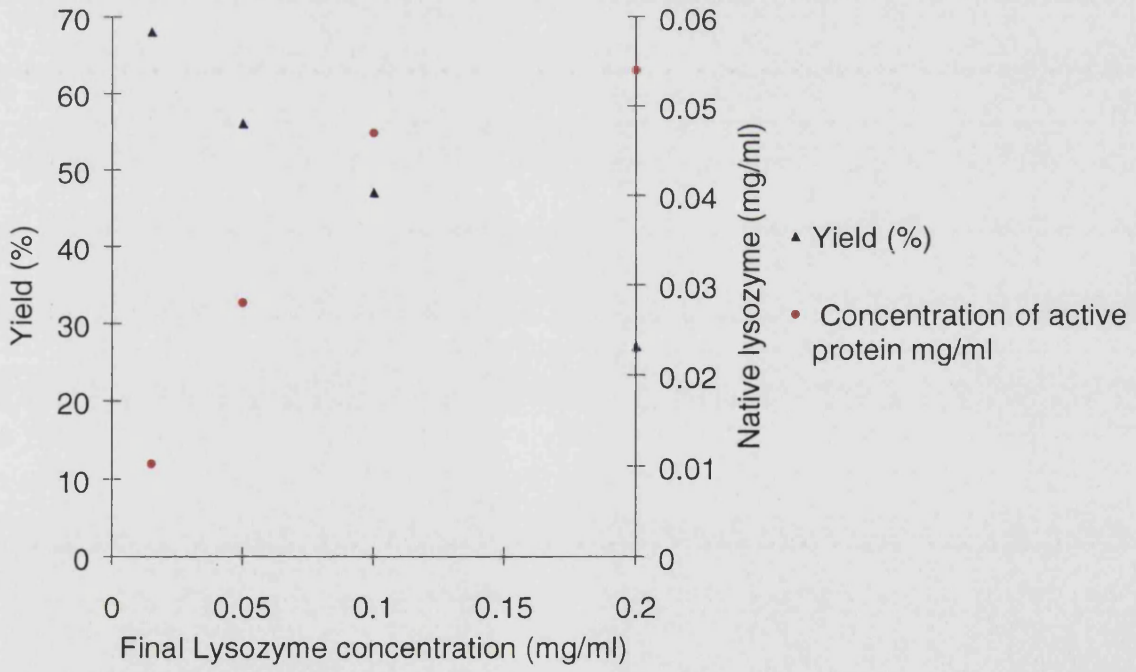

Figure 6-1 The effect of denatured lysozyme concentration on the yield and concentration of native lysozyme.

This result is significant with respect to the production of proteins via the refolding method. Large costs are associated with dealing with large process

stream volumes so a concentrated product stream is preferred over a dilute one. In refolding this must be balanced against the increase in fermentation costs as the yield of the process decreases.

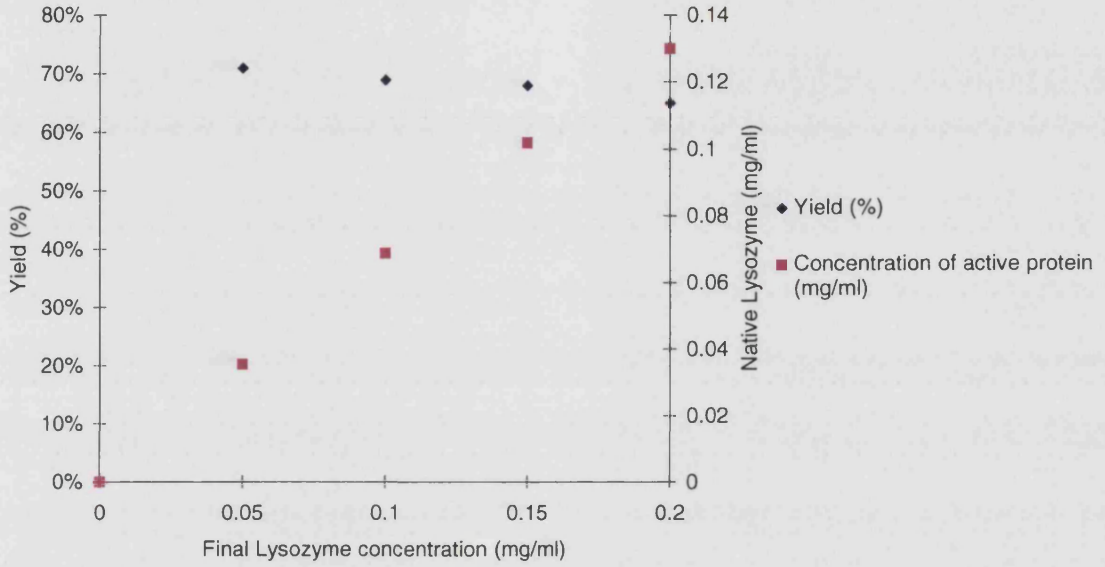

Figure 6-2 The effect of denatured lysozyme concentration on the yield and concentration of native lysozyme during continuous refolding.

Applying the same format to data obtained for the refolding of lysozyme via the continuous process a very different picture is seen. Assuming that protein is added over a twenty minute period the concentration of denatured protein in solution at any time varies from 0.0025 mg/ml to 0.01 mg/ml. Thus, although the final concentration of lysozyme is the same, the concentration of denatured protein at any one time never exceeds the concentration at which aggregation predominates.

It is likely that many refolding processes are not operated in areas where the yield is highest as it is preferable to produce a high concentration of protein rather than to produce a large volume of dilute protein. Datar *et al.* (1993) have assessed the cost of individual process units for the refolding of tissue plasminogen activator. Seventy five per cent of the capital cost of the process

can be attributed to the refolding tanks. In the same paper it was shown that with a refolding yield of 20% and final concentration of 2.4 μg/ml that 10 refolding tanks of 180 m^3 would be required to produce 11 kg of tissue plasminogen activator per year via refolding. By maintaining the same yield and increasing the concentration of refolded protein to 4.8 μg/ml the total volume of fluid processed in the refolding tanks could be reduced from 23,000 m^3 per year to 11,500 m^3 per year. This is a good indicator of why it is so important to increase both the yield and concentration of protein being refolded.

However, it is not possible to assess the impact of yield versus concentration on the process cost simply by considering the refolding tanks. The effect of the yield and concentration of refolded protein on cost must be considered over the entire process. As the concentration of protein in the refolding tanks changes the size of the tanks and ancillary equipment will change.

6.1 Methodology

This appraisal involves assessing the impact of varying the concentration of lysozyme in the refolding tanks on a defined process. The effect of this change on the cost of all major pieces of process equipment is assessed. In addition to this work, the same analysis is performed to an identical process but with a recycle stream introduced. The recycle takes aggregated material from the refolding tanks and recycles it to the solubilisation tanks.

Datar *et al.* (1993) stated that "Unfortunately biochemical engineers, unlike their counterparts in chemical engineering, are not yet able to fall back on well established thermodynamic and physical principles to simulate optimisation of bioprocesses. As a consequence extensive pilot plant studies play a central role.....".

Using the data and model from the previous chapter it is the aim of this Chapter to simulate the optimisation of lysozyme refolding by using the

reaction engineering model formulated in Chapter 5. To optimise the total cost the operating system needs to be defined. The process configuration used in this study is shown in Figure 6-3

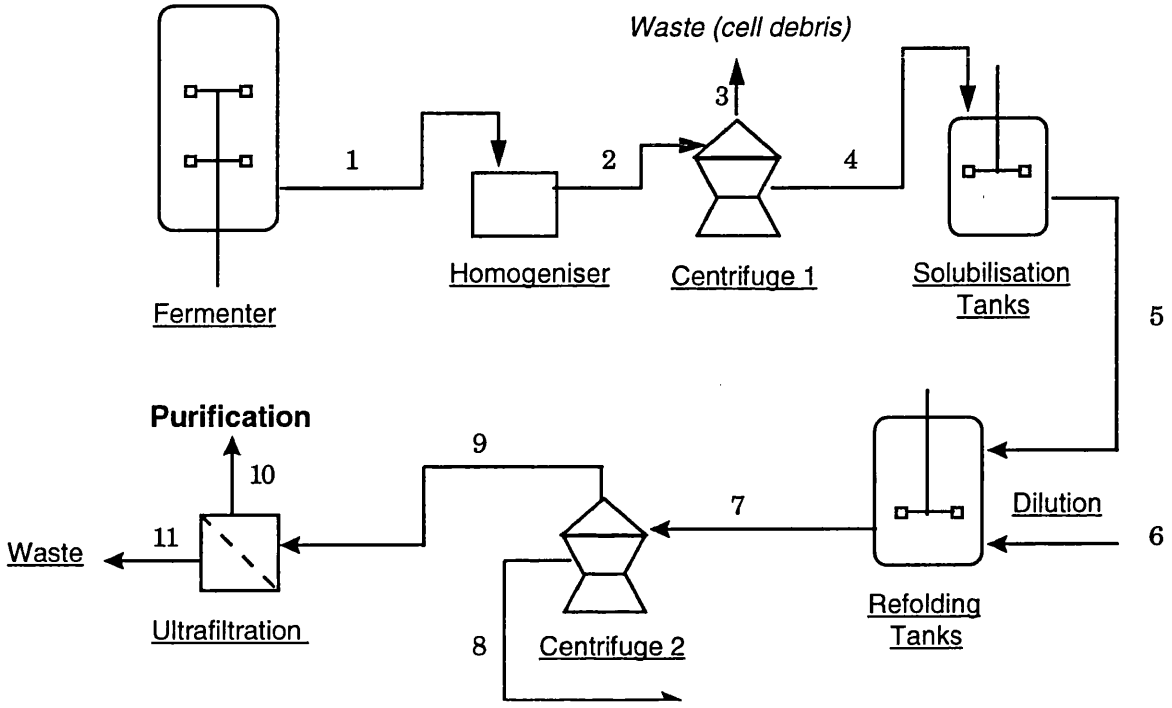

Figure 6-3 Process diagram used for the economic assessment of the refolding of lysozyme

This is a typical process flowsheet for the refolding of recombinant proteins. (Datar *et al.* (1993); Middleberg (1996)). Fermentation media are prepared and sterilised in a continuous steriliser. *E.coli* cells are grown in a seed fermenter and then transferred to the main fermenter. The cells are grown at 37°C and the time required for the fermentation (including turnaround) is approximately 24 hours (Petrides *et al.* (1995). The final concentration of cells (dry cell weight) in the fermenter is 30-35 g/l (Petrides *et al.* (1995), Datar *et al.* (1993), Petrides *et al.* (1989)). Once the fermentation is complete the cells are killed using a thermal (Petrides *et al.* (1995)) or chemical (Petrides *et al.* (1989)) sterilisation process. A high pressure homogeniser is used to rupture the cells and release the inclusion bodies. Once the cells are ruptured the

inclusion bodies are separated from the rest of the cell debris using a disk stack centrifuge. Typically a disk centrifuge operating with intermittent discharge can produce a sludge of 40-50 g/l (Petrides *et al.* (1989)). The inclusion bodies are then dissolved in the solubilisation tanks. Inclusion bodies are generally dissolved in 6M GuHCl or 8M urea and 0.2M β-mercaptoethanol (Petrides *et al.* (1995)). β-mercaptoethanol is used in preference to dithiothreitol as it is considerably cheaper (Middleberg (1996)). The residence time in the reactors is generally 2-8 hours (Petrides *et al.* (1995), Datar *et al.* (1993)). The reduced denatured protein then passes to the refolding tanks. Refolding takes place in the refolding tanks. The residence time of the protein in the refolding tanks varies considerably from 2 hours for porcine growth hormone (Petrides *et al.* (1989)) to 12 hours for human proinsulin (Petrides *et al.* (1995)) to 48 hours for tissue plasminogen activator (Datar *et al.* (1993)). After refolding the aggregated material is removed from the process stream using another centrifuge. In processes where the aggregated material is not recycled a depth filter may be used in preference to a centrifuge. The refolded protein is concentrated using ultrafiltration membranes in readiness to be sent to the final purification step. Equipment such as sterilisers, compressors, seed fermenters, pumps, heat-exchangers etc. are not considered as they represent only a small proportion of the total cost.

The total annual cost, A_T, of a project can be described by the following equation.

$$A_T = A_{FC} + A_{CON} + A_{UT} \qquad \text{Equation 6-1}$$

Where A_{FC} is the fixed capital cost, A_{CON} is the annual cost of consumables, such as fermentation media and A_{UT} is the utilities and labour costs. In this assessment only the fixed capital cost of the equipment is considered. The aim of this assessment is to calculate the effect of different refolding

conditions on the process equipment, any reduction in the process volume will lead to a reduction in the cost of consumables. It can be assumed that the utilities and labour costs will not vary significantly depending on whether the process is run at high yield or high concentration.

The fixed capital cost can be calculated by performing a mass balance over the system and calculating the size of the major components. The associated fixed costs like land, taxes, buildings, surveys, legal fees, piping, freight, insurance, instrumentation, utilities and construction are not considered. The associated fixed costs are a function of the total fixed capital cost and would not reflect how different refolding conditions affect the cost of individual items.

The mass balance is performed by assuming a given production rate of 100,000 kg per annum. This is the equivalent of the annual US market for bovine growth hormone in 1991 (Datar *et al.* (1993)). The cost and losses associated with the final chromatographic purification step are not assessed. The concentration of protein sent to the final purification step is set a 1g/l. This is the concentration of tissue plasminogen activator quoted by Datar *et al.* (1993) that is sent to an IgG-Sepharose column. The calculation of the mass balance was performed using a spreadsheet (Microsoft Excel).

The mass balance is calculated by using the required production rate and back calculating the mass flow-rates involved in each individual step. A brief description of the logic involved is presented :

- Given the required production rate the amount of native protein required to be produced in the refolding tanks can be calculated.

- By setting the concentration of refolding lysozyme the yield can be calculated using the model proposed in Chapter 5. This calculates the concentration of lysozyme in the refolding tanks. The residence time in the

refolding tanks is taken as 40 minutes. The concentration of lysozyme in the refolding tanks is varied to give different process costs.

- As the production rate has been stated to be 100,000 kg/yr, the flow-rate from the refolding tanks to the second centrifuge and the ultrafiltration unit can be calculated. It is assumed that no active protein is lost in either the centrifuge or during ultrafiltration. These assumptions are valid as the flow-rate of the material recovered is insignificant compared to the flow-rate from the centrifuge to the ultrafiltration unit and binding of proteins to ultrafiltration membranes at low protein concentration is generally insignificant.

- By setting the concentration of denatured reduced protein in the solubilisation tank at 10 g/l the flow-rate into and out of the solubilisation tank can be calculated. For the sake of this simulation it is assumed that the centrifuges give a constant concentration of recovered protein. In practise a constant concentration of protein to the refolding tanks could be achieved by the on-line monitoring of the protein concentration in the refolding tanks. The assumption that the concentration of reduced protein is constant allows the calculation of the diluant stream to the refolding tanks.

- Assuming losses of product in centrifuge 1 and homogeniser of 80 and 90 percent respectively the mass production rate in the fermenter can be calculated. It is then assumed that the fermenter can produce 35 g/l dry cell weight of cells. Assuming that 20% of that mass is inclusion body protein, that the inclusion bodies are 80% lysozyme and that the residence time in the fermenter is 24 hours, the size of the fermenter can be calculated. This gives the flow rate into and out of the homogeniser and as the concentration of product leaving the centrifuge is fixed at 10 g/l the final process stream can be deduced.

The size of the fermentor, solubilisation tanks and the refolding tanks are a function of the flow-rate into each vessel and the residence time associated with each vessel. The homogeniser and the ultrafiltration units are sized according to their throughput and the centrifuges are sized according to the flow-rate required and the desired recovery of solids.

The cost of each unit is based upon the costs from a paper by Petrides *et al.* (1995). It was thought best to take all the cost data from one source rather than from different sources. The cost is in dollars and the prices are based on the cost of equipment in 1994.

6.2 Individual Unit Costs

6.2.1 Ultrafiltration Unit

The cost of the ultrafiltration unit, A_U, is directly proportional to the area of membrane, a_M, required and the cost of the membrane a_C.

$$A_U = a_C . a_M \qquad \textbf{Equation 6-2}$$

Assuming that there is a linear relationship between flux, J, and membrane area

$$a_M = \frac{Flow\ in - Flow\ out}{J} \qquad \textbf{Equation 6-3}$$

The flux and the concentration of product will be specified at 50 l/m^2h (Petrides *et al.* (1995)) and 1mg/ml respectively. a_C is calculated from Petrides *et al.* (1995) to be \$1444 m^{-2}.

6.2.2 Centrifuge 2

The cost of a centrifuge, A_{C2}, is directly related to its total settling capacity, Σ. The settling capacity is a function of the flow-rate through the centrifuge, Q, and the desired recovery of the precipitate. Q/Σ has been calculated

experimentally for the separation of inclusion bodies from cell debris as 10^{-9} m/s (Mannweiler *et al.* (1989). No data can be found for the value of Q/Σ for refolding aggregates so a value of 10^{-6} m/s was assumed. 10^{-6} m/s is the value calculated for the recovery of yeast cells from fermentation broth (Mannweiler *et al.* (1989)). Refolding aggregates are visible to the eye and are considerably larger than inclusion bodies and yeast cells so 10^{-6} m/s can be seen as a "worst case" assumption. Q is known from the mass balance. This allows calculation of Σ.

$$\frac{Q}{10^{-6}} = \Sigma \qquad \textbf{Equation 6-4}$$

The cost of the centrifuge can then be calculated from the data from Petrides et al. (1995). They use a value of \$1.88 m^{-2}. The cost of the centrifuge, A_C, is given by:

$$A_C = 1.88\Sigma \qquad \textbf{Equation 6-5}$$

6.2.3 Refolding Tanks

The volume of the refolding tanks, V_R is directly proportional to the concentration of protein being refolded. The cost of the refolding tanks, A_R, is directly proportional to the volume of the tank. There are several tanks of various size listed in Petrides *et al.* (1995) so they were plotted and a linear regression performed.

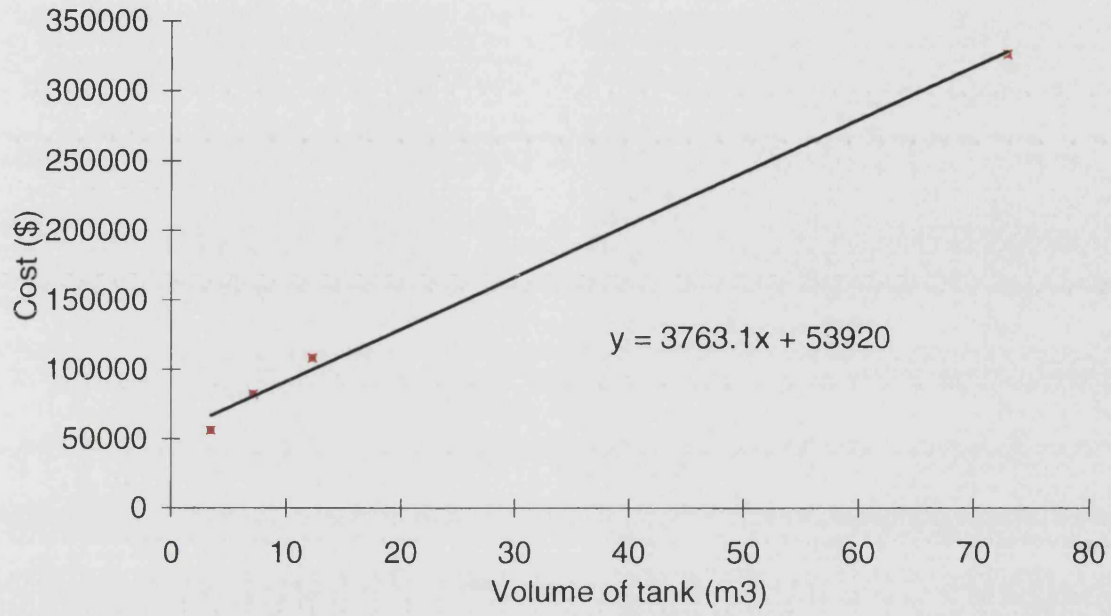

Figure 6-4 Linear Regression for the calculation of the cost of refolding tanks

This gives the cost of the tank, A_R, as:

$$A_R = 3763V_R + 53920$$ **Equation 6-6**

6.2.4 Solubilisation Tanks

The size ,V_S, and cost, A_S, of the solubilisation tanks is directly proportional to the concentration of denatured protein, C_S. The cost of the solubilisation tanks are calculated using the same linear regression as used for the refolding tanks.

$$A_S = 3763V_S + 53920$$ **Equation 6-7**

6.2.5 Centrifuge 1

The cost of a centrifuge is directly related to its total settling capacity, Σ and is calculated in the same manner as previously described. For the 90% recovery of inclusion bodies using a Westfalia disc-stack centrifuge Mannweiler calculated the value of Q/Σ to be 10^{-8}. Q is known from the mass balance. This allows calculation of Σ.

$$\frac{Q}{10^{-6}} = \Sigma \qquad \textbf{Equation 6-8}$$

The cost of the centrifuge can then be calculated from the data from Petrides et al. (1995). They use a value of \$1.88 m^{-2}. The cost of the centrifuge, A_C, is given by:

$$A_C = 1.88\Sigma \qquad \textbf{Equation 6-9}$$

6.2.6 Homogeniser

The homogeniser cost, A_H, is directly related to the flow-rate of fluid passing through it, Q. The cost takes into account 4 discrete passes. This ensures at least ninety percent disruption of the cells. From Petrides *et al.* (1995).

$$A_H = 13.46\,Q \qquad \textbf{Equation 6-10}$$

6.2.7 Fermenter

It has previously been described how the mass of lysozyme needed to be produced in the fermenter is calculated. It is then assumed that the fermenter can produce 35 g/l dry cell weight of cells. Assuming that 20% of that mass is inclusion body protein, that the inclusion bodies are 80%

lysozyme and that the residence time in the fermenter is 24 hours, the size of the fermenter can be calculated. The cost of the fermenter, A_F, is based on the volume of the fermenter, V_F, calculated directly from the cost per unit volume determined by Petrides *et al.*(1995)

$$A_F = 15285.V_F$$ **Equation 6-11**

6.3 Results and Discussion

6.3.1 The effect of lysozyme concentration in the refolding tank on the fixed capital cost

The effect of concentration of lysozyme on the fixed capital cost of each piece of equipment will be studied individually. The range of concentration of refolding lysozyme studied is 0.05 g/l to 2 g/l.

6.3.1.1 Ultrafiltration, Centrifuge and Refolding Tanks Costs

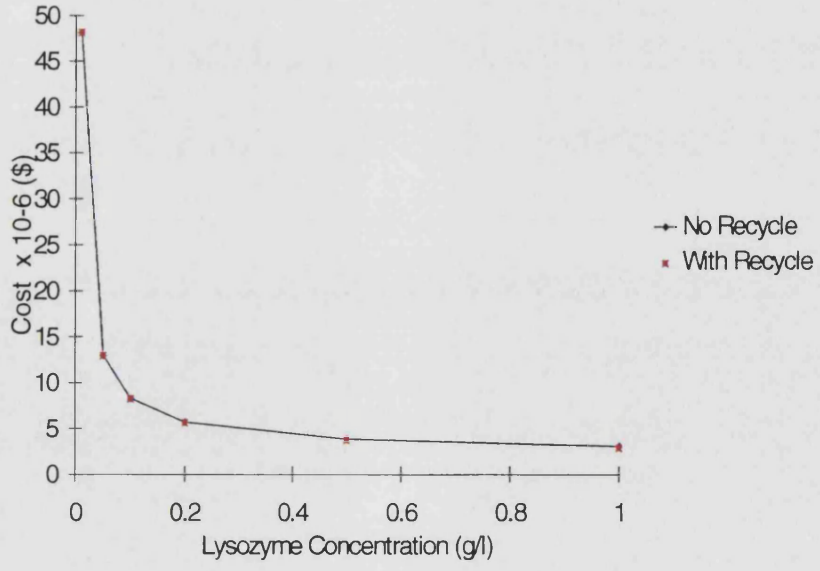

Figure 6-5 The effect of lysozyme concentration in the refolding tank on ultrafiltration cost

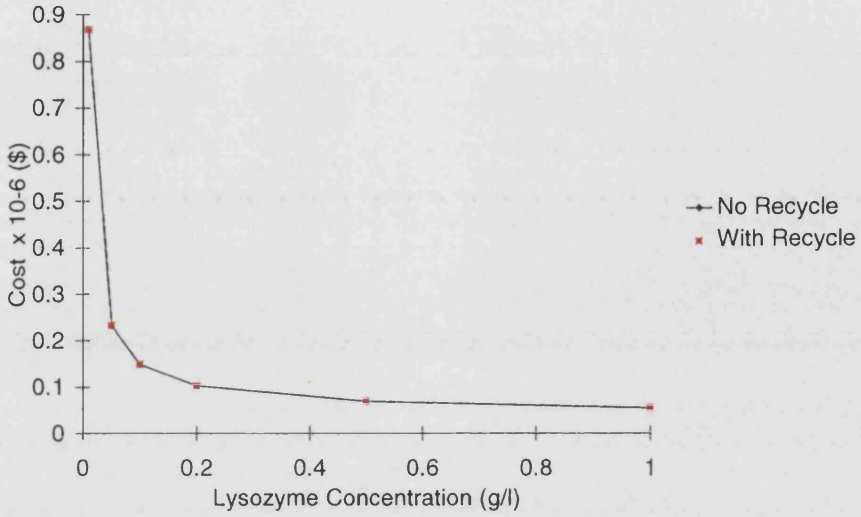

Figure 6-6 The effect of lysozyme concentration in the refolding tank on centrifuge2 cost

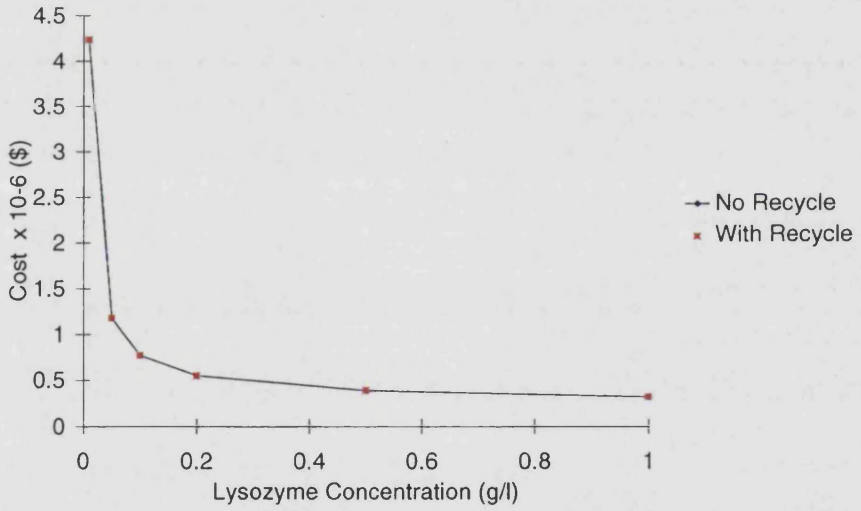

Figure 6-7 The effect of lysozyme concentration in the refolding tank on the refolding tanks cost

The effect of concentration on the cost of the ultrafiltration unit, centrifuge 2 and refolding tanks is the identical. This is because the flow through the ultrafiltration unit and centrifuge is directly proportional to the fermenter volume. As the concentration in the refolding tanks is increased there is an exponential decrease in the cost of each of the units. The recycling of

aggregates has no effect on the cost of these units. This is because these units are sized on the concentration of soluble active protein. In this simulation the recycle does not have an effect on the mass flow rate of active lysozyme and therefore no difference in cost is observed. The increase in size of the refolding tanks if this were to be taken into account would be equal to the flow-rate in the recycle line multiplied by the residence time needed in the refolding tanks. The centrifuge would increase in size correspondingly. The volumetric flow to the ultrafiltration units would not change.

6.3.1.2 Solubilisation Tanks

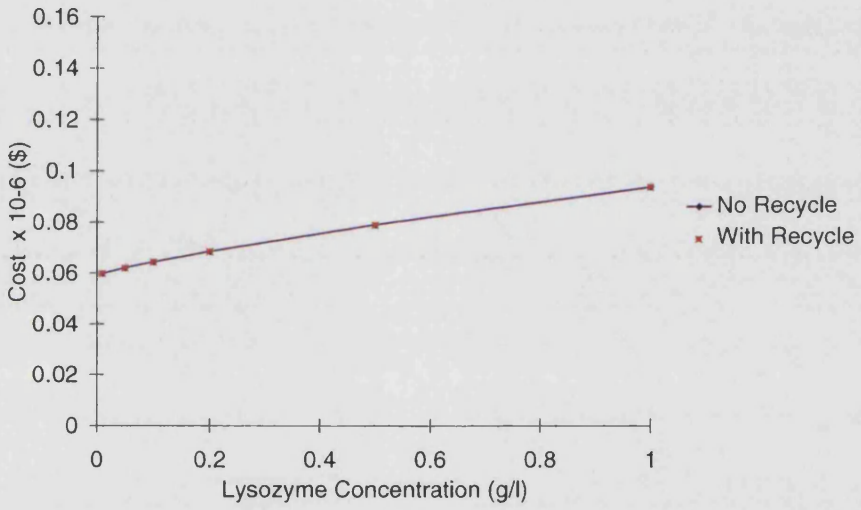

Figure 6-8 The effect of lysozyme concentration in the refolding tank on the solubilisation tanks cost

The cost of the solubilisation tanks increases as the concentration of protein in the refolding tanks is increased. This is because as the concentration increases the yield increases and a greater amount of reduced protein has to be added to the system to achieve the same production rate of refolded protein. The recycle has no effect on the size of solubilisation tank. This is because irrespective of where the reactant (denatured protein) is coming from, inclusion bodies or refolding aggregate, the same mass is needed to give a

specified concentration of active protein in the refolding tanks regardless of whether there is a recycle or not.

6.3.1.3 Centrifuge 1, Homogeniser and Fermenter Costs

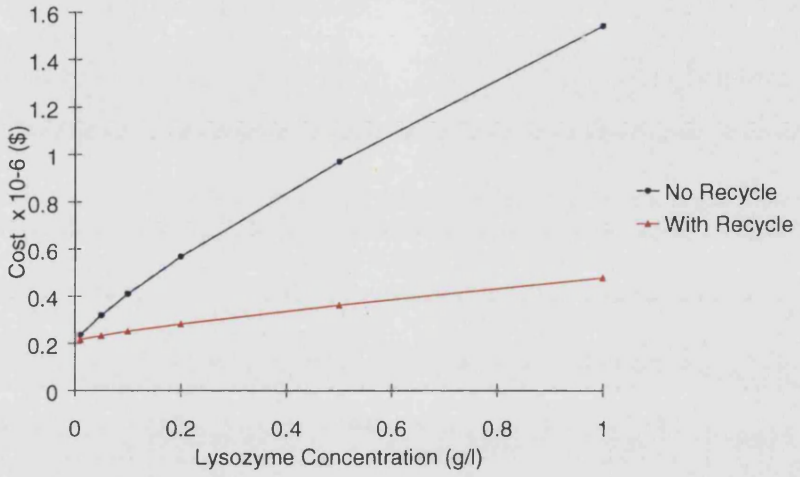

Figure 6-9 The effect of lysozyme concentration in the refolding tank on centrifuge 2 cost

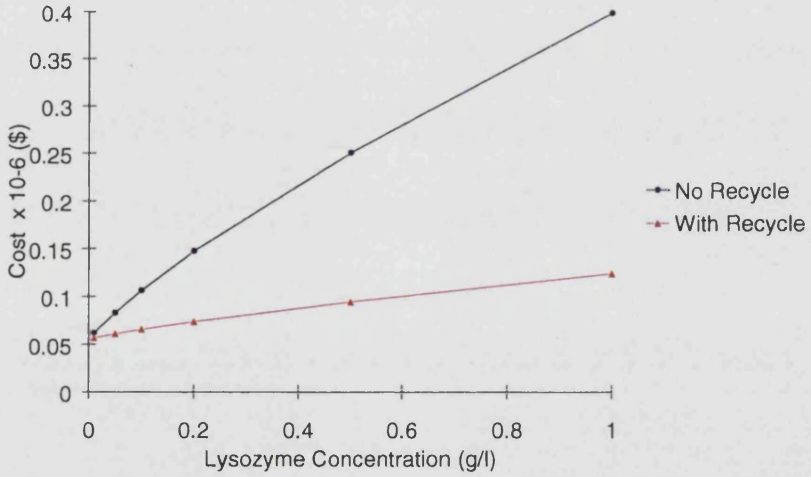

Figure 6-10 The effect of lysozyme concentration in the refolding tank on the homogeniser cost

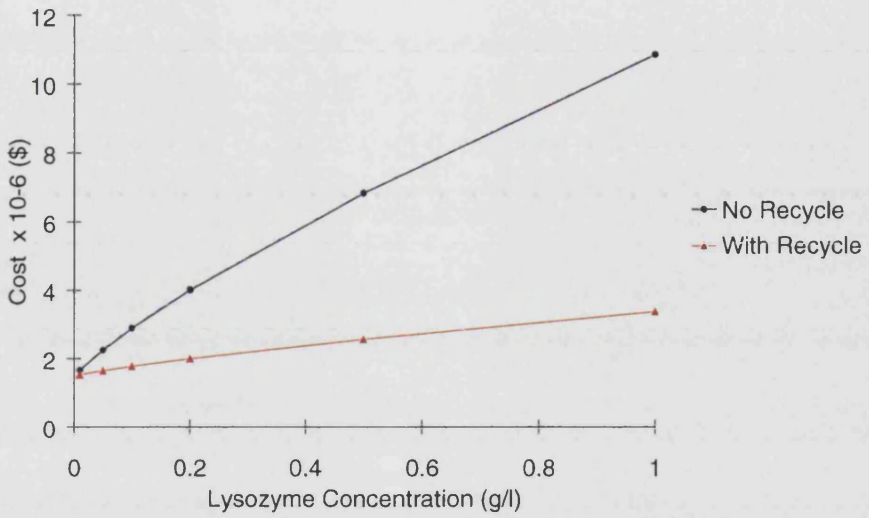

Figure 6-11 The effect of lysozyme concentration in the refolding tank on fermenter cost

The effect of the concentration of lysozyme in the refolding tanks on the cost of centrifuge 1, the homogeniser and the fermentor are the same. This is because the cost of the centrifuge1 and the homogeniser are directly proportional to the size of the fermenter. As the concentration of lysozyme in the refolding tanks increases the cost of the fermenter increases. As the concentration increases the yield drops and more inclusion body protein has to be produced in the fermenter. Therefore the size and cost of the fermenter increases, increasing the cost of the homogeniser and centrifuge 1.

The recycle significantly decreases the cost of the fermenter, homogeniser and centrifuge 1. This is because as the yield in the refolding tanks decreases the increase in the production of inclusion body protein necessary is decreased by the amount of protein being recycled. This effectively increases the yield of the overall process.

The effect of concentration of lysozyme in the refolding tanks and the effect of recycling refolding aggregates on the cost of individual units has been assessed. It has been shown that increasing the concentration of lysozyme in

the refolding tanks increases the cost of units prior to refolding. Concomitantly, the refolding tanks, centrifuge 2 and ultrafiltration unit decrease in cost. The recycling refolding aggregates has no effect on the cost of the solubilisation tanks, the refolding tanks, centrifuge 2 and ultrafiltration unit. However the cost of the fermenter, homogeniser and centrifuge 1 are all significantly reduced. This work shows that the optimal concentration of lysozyme in the refolding tanks will involve a trade off of the cost of units prior to refolding and those after solubilisation. It also demonstrates that although recycling of aggregates does not effect units after centrifuge 1 it will decrease the overall purchase cost of the equipment.

6.3.2 Total Cost and Comparison of Unit Costs

The effect of concentration of lysozyme in the refolding tanks on the cost of individual units has been discussed. The analysis of individual units gives an insight into the optimal concentration of lysozyme in the refolding tanks. In this section the costs of the individual units are compared and the optimal concentration of lysozyme in the refolding tanks calculated. Using a spreadsheet model the concentration of lysozyme in the refolding tanks was varied until the total fixed cost was minimised. (Microsoft Excel Solver uses a Generalised Reduced Gradient (GRG2) non-linear optimisation code. Linear and integer problems use the simplex method with bounds on the variables, and the branch-and-bound method.)

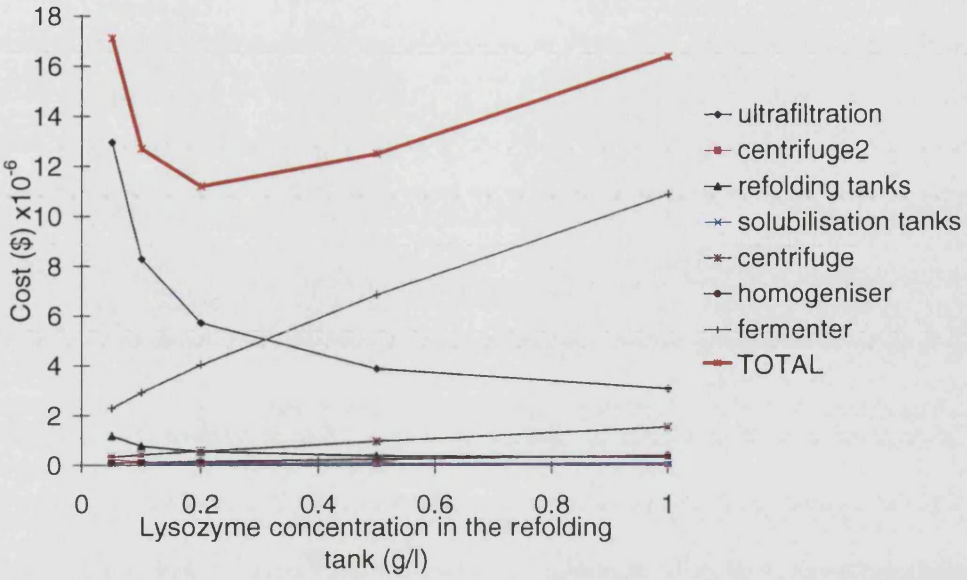

Figure 6-12 The effect of lysozyme concentration on the capital cost of individual process equipment and the total A_{FC}.

Figure 6-12 shows the effect of lysozyme concentration on the capital cost of individual process equipment and the total A_{FC}. It can bee seen quite clearly that a minimum cost occurs at approximately 0.2 mg/ml. The minimum cost was found to be at 0.22 mg/ml. This corresponds to a refolding yield of 35% and a cost of $\$11 \times 10^6$. Middleberg (1996) has suggested that for competing refolding and aggregation reactions in a continuous stirred-tank reactor where there are no intermediates in the refolding pathway, high conversion minimises the annual cost. This has been shown not to be true for the refolding of lysozyme which does not have any intermediates in its refolding pathway (Ptitsyn *et al.* (1990)).

Middleberg (1996) uses the cost capacity method of cost analysis which is based on equations similar to Equation 6-12.

$$Cost = 10^{\alpha}.Volume^{\beta} \qquad \textbf{Equation 6-12}$$

This method is extremely sensitive to the values of α and β. Middleberg (1996) uses this analysis in an attempt to derive a dimensionless analysis of process cost. Values of α for the solubilisation tanks of 7.00 and 4.17 for the refolding tanks respectively were used. The β values are 0.6 for the solubilisation tanks and 0.54 for the refolding tanks. Using these values the solubilisation tanks are approximately 1000 times more expensive per m^3 of tank than the refolding tanks. Petrides *et al.* (1995) found only a five fold difference in the cost per cubic metre. This represents of the economy of scale and would seem more accurate.

Figure 6-12 shows that the two most important costs for the refolding of lysozyme are the fermentation and ultrafiltration costs. At low refolding concentrations the cost of concentrating the protein is extremely high, the yield is so fermentation costs are at a minimum. As the refolding concentration is increased less membrane area is required to concentrate the lysozyme to 1 g/l. However, as the concentration is increased the yield of the process is decreased and therefore the size and cost of the fermentor increases. For the refolding of tissue plasminogen activator (tPA) the refolding tanks represented the single most important cost, accounting for 75% of the fixed capital cost. The reason for the difference is due to the time taken for refolding. tPA requires 48 hours to refold efficiently and refolding is performed at 2.4 mg/l (Datar *et al.* (1993)). This is 10 times more dilute than the optimal concentration for the refolding of lysozyme and the residence time in the refolding tanks is 72 times longer than the residence time for lysozyme. Therefore the refolding tanks necessary to produce the same amount of tPA as lysozyme would be approximately 720 times bigger. The rate constants for refolding and aggregation of tPA have not been calculated so a direct comparison of the costs of the two processes is not possible.

6.3.3 Total Cost and Comparison of Unit Costs with Recycle.

Lysozyme refolding aggregates have to be removed from the process prior to the final purification. It is has been suggested that it would be economically advantageous to recycle these aggregates. Resolubilisation and the subsequent refolding of protein aggregates has been shown to be possible by DeYoung *et al.* (1993). Assuming that 80% of the aggregates can be recovered the same analysis of A_{FC} is performed as previously explained.

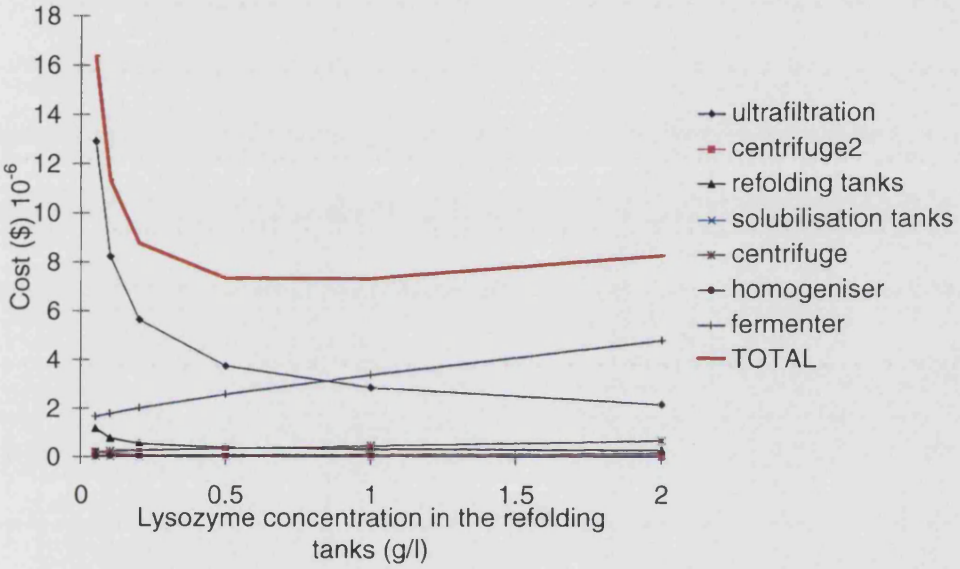

Figure 6-13 The effect of lysozyme concentration on the capital cost of individual process equipment and the total A_{FC} with recycle of refolding aggregates.

Figure 6-13 shows the effect of lysozyme concentration on the capital cost of individual process equipment and the total A_{FC} with recycle of refolding aggregates. The optimum concentration of lysozyme in the refolding tank is calculated to be 0.717 g/l. As has already been shown this is because the fermentation costs do not increase as rapidly as in the case without recycle. The minimum cost in this case is $\$7 \times 10^6$. This is a significant reduction on the $\$11 \times 10^6$ A_{FC} without recycle.

Table 4 The difference in cost of major pieces of equipment for the refolding of lysozyme with and without recycle of refolding aggregates

Piece of Equipment	Cost without Recycle $ x10^6	Cost with Recycle $ x10^6	Difference $ x10^6
Ultrafiltration	5.4	3.2	-2.2
Centrifuge 2	0.09	0.06	-0.03
Refolding Tanks	0.53	0.35	-0.18
Solubilisation Tanks	.07	.09	+.02
Centrifuge 1	0.60	0.42	-0.18
Homogeniser	0.16	0.11	-0.05
Fermenter	4.2	3.0	-1.2
Total	11.1	7.3	-3.8

Table 4 Table 1epresents a breakdown of the difference in purchase cost of major pieces of equipment for the refolding of lysozyme with and without recycle of refolding aggregates. With recycle there is a saving on all pieces of equipment except the solubilisation tanks.

It should be noted that despite the obvious economic advantages of recycling aggregated material, that there are several regulatory aspects which would need to be addressed if this process were to be introduced. In theory it is possible for a protein molecule to be recycled infinitely in the recycle loop. It is difficult to predict what chemical modifications the protein may undergo after being recycled several times. By-products created by side-chain reactions would have to be removed. However, despite there being an increased chance of modification during recycling, the process for single step refolding already takes steps to remove any modified proteins using methods such as ion exchange and more appropriately affinity chromatography. Therefore regulatory issues should be of no significant concern.

6.4 Conclusions

Using the model described in section 5 it has been shown that the optimal conditions for refolding are not necessarily at low concentration and high yield when considering the total fixed cost of the process. It has been shown that for the refolding of lysozyme that the optimal cost of a conventional process is \$11 x10^6. This corresponds to a refolding concentration of 0.22 g/l. It was also proposed that the cost could be reduced by recycling aggregates formed in the refolding step to the solubilisation tanks. This effectively increases the overall process yield even though the refolding yield is low. The optimal refolding concentration with recycle of the aggregates was found to 0.72 g/l. The minimum cost was \$7 x10^6 representing a saving of 35% on the original estimate. Recycling of aggregates is not a protein specific solution and would improve the efficiency of any refolding process.

7. Chaperone Assisted Refolding of Lysozyme

Molecular chaperones have been shown to enhance the *in vitro* refolding of a number of proteins. The proteins studied are usually large proteins which give very poor refolding yields in the absence of chaperones. In this Chapter the effect of GroEL, an *E.coli* chaperone, on the refolding of lysozyme is investigated. The range of denatured lysozyme concentration studied is 0.015 mg/ml to 0.2 mg/ml. The use of molecular chaperones on an industrial scale has not been investigated. To make such a process economically viable it has been suggested that the chaperones used would have to be recovered and recycled. In this Chapter the recovery of GroEL from the refolding buffer is investigated using ultrafiltration membranes. The effect of the process on the effectiveness of GroEL as a refolding enhancer is also studied.

7.1 Introduction

The general concept of molecular chaperones was developed as a result of studies on the biogenesis of ribulose bisphosphate carboylase-oxygenase (Ellis (1990)). Since their discovery, *in vivo*, there has been great interest in the potential use of molecular chaperones for protein folding *in vitro*. Molecular chaperones do not catalyse refolding, they prevent the polypeptide chain from forming incorrect associations with itself and other proteins (Hendrick and Hartl (1993)). Chaperones are not substrate specific, binding to a number of different polypeptides. They do not bind to the native form of the protein, they are not components of the final structure and they are conserved throughout eukaryotic and prokaryotic organisms.

It should be noted that chaperones are significant in roles other then correct folding. They limit damage caused by stress such as heat shock and are often preferentially expressed by the cell under such conditions. This led to them being known in eukaryotic systems as heat-shock proteins. However, chaperones are expressed in abundance under non-stressful conditions and

are essential for the normal operation of a healthy cell. (Linquist and Craig (1988), Fayet *et al.* (1989)). Chaperones are involved in intracellular transport, unassembling and reassembling proteins as they pass through cellular membranes. (Linquist and Craig(1988), Fayet *et al.* (1989), Beckman *et al.* (1990)). They are involved in the dissolution of protein aggregates (Pelham (1986)) and in the degradation of proteins with high turn-over rates.

There are three main classes of chaperones. These are known as the Stress 90, Stress 70 and Stress 60 families. Stress 60 proteins are also known as chaperonins. These three groups by no means cover all proteins which are involved in protein refolding. Other helper proteins include protein disulphide isomerase (PDI) which enhances the rates of disulphide formation, reduction and rearrangement (Ellis and van der Vies (1991), Freedman (1991)), and prolyl peptide isomerase which catalyses peptide bond isomerisation (Goldenberg (1992)).

7.1.1 Stress 70 proteins

Stress 70 proteins have been studied for many years in relation to their overexpression during shock (Linquist and Craig (1988), Fayet *et al.*. (1989)). For example, accumulation of unfolded protein in *E.coli* leads to the increased synthesis of DnaK (Wickner *et al.* (1991)). More recent work has illustrated their importance in reassembly and degradation processes. Stress 70 proteins bind to polypeptides which are in a highly unfolded or a misfolded state. It has been suggested that they bind to primary structure sequence rather than secondary and tertiary structural motifs. Proteins bound to Stress 70 chaperones do not refold, once released from the chaperone the protein can refold, aggregate, be passed onto a different chaperone or rebind to a Stress 70 protein. Under normal conditions aggregation is rare, 98% of protein translocated successfully refolds.

Stress 70 proteins are highly conserved through evolution. *E.coli.* DnaK shares 50% sequence homology with mammalian cytosolic hsp 70,

mitochondrial Ssc1p and the endoplasmic reticulum proteins Kar2p and BiP. The first 450 N-terminal amino acids are more highly conserved than the C-terminal end. The C-terminal is considered to be the region of the protein which confers specificity, giving rise to the different binding affinities found in different Stress 70 proteins. Despite this limited specificity DnaK will bind foreign proteins expressed in *E coli*.

7.1.2 Stress-60

These proteins are found in prokaryotic cells and organelles of eukaryotes such as mitochondria (Ellis and Hemmingsen (1988)). The quaternary structure of Stress 60 proteins has been revealed by electron microscopy (Hendrix (1979), McMullin and Halberg(1988)). They are large oligomers with 14 subunits, each unit being approximately 60 kDaltons arrayed as two stacked rings of 7 subunits. In *E coli* GroEL interacts with another protein called GroES. GroES is an oligomer of seven subunits each approximately 10 kDaltons arranged in a seven membered ring (Chandrasekhar *et al.*. (1986)). Mitochondria contain a structurally and functionally homologous protein.

Stress 60 protein synthesis increases under stress conditions. Similarly Stress 60 proteins exhibit high ATP affinity and weak ATPase activity. They appear to bind to folding intermediates preventing aggregation and when ATP and GroES are present they promote the polypeptide to refold. In all cases it has been shown that K^+ atoms are necessary for the release of the native polypeptide from the GroEL complex. When first discovered it was thought that chaperonins could accelerate refolding rates but experiments have shown that chaperonins do not act as catalysts (Jaenicke (1993), Miller *et al.* (1993)). They act as aggregation or misfolding inhibitors. *In vitro* studies have shown that GroEL can inhibit the aggregation of several unfolded proteins which are prone to aggregation (Buchner *et al.* (1991), Martin *et al.* (1991)).

The possible complexes formed between the two chaperones and their affinity for the protein being refolded have been studied in some detail (Staniforth *et*

al. (1994)). From this work it has been shown that from the possible complexes, cpn 60 binds the target protein tightest and the cpn60-ATP-cpn10 binds the weakest. A general scheme has been proposed for Stress 60 action.

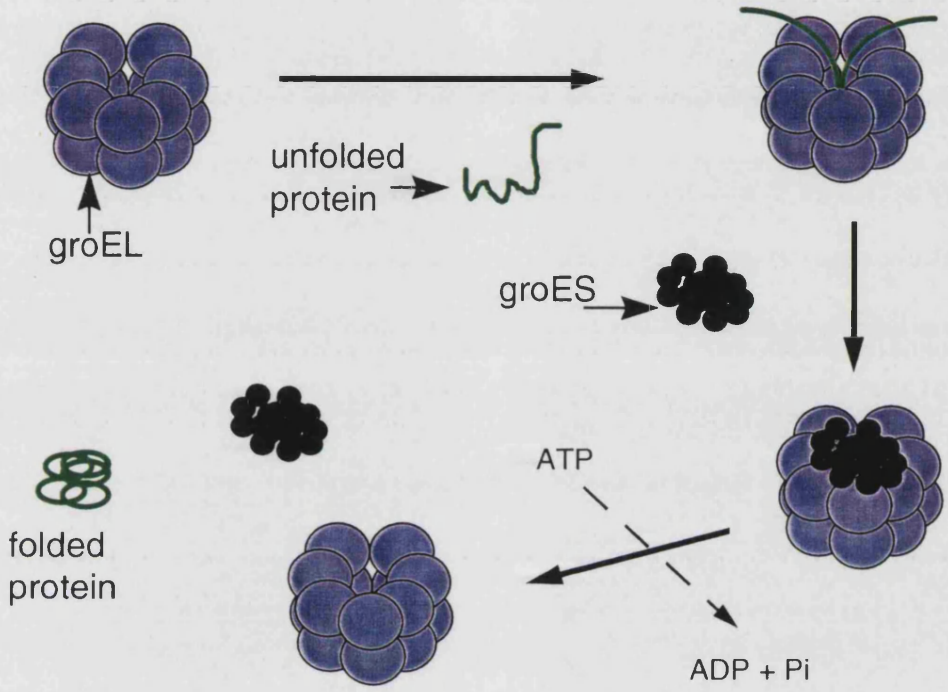

Figure 7-1 Represents the mechanism of action for the E.Coli chaperonins GroEL and GroES.

1. The substrate binds to the GroEL complex. There are seven binding sites located on the inside of the protein cavity. One binding site per monomer. The exact position of the protein on or in the chaperone is still not resolved (Hendrick and Hartl (1993) Martin *et al.* (1991), Staniforth *et al.* (1994)).

2. GroES binds to the opposite side of the GroEL protein.

3. Partial folding of the polypeptide takes place in close association with the GroEL protein. This process is ATP dependant.

4. The protein is then released into the bulk phase where it can refold or rebind. GroEL complexes do not bind to folded proteins.

Release is thought to occur when the protein reaches a state whereby the unfolded structural motifs that the chaperonin recognises change, to the extent that the affinity of the chaperone for the "molten globule" becomes insignificant. An alternative explanation is that ATP binding to the Stress 60-Stress 10-protein complex reduces the affinity of the Stress 60 protein for the folding protein and thus facilitates release (Staniforth *et al.* (1994)). If the protein does not proceed along its folding pathway it may then rebind to another Stress 60 protein.

More recent work has shown that refolding may well occur inside the cavity of the GroEL ring (Hartl (1996). This model is known as the Anfinsen cage model and has been compared to a single protein molecule refolding in a test-tube. This model suggests that protein refolds to a stable form in the GroEL cavity and is released in a form which cannot aggregate. The proposed mechanism of interaction between ATP, GroEL and GroES is as described earlier the only difference in the two models is the state in which the protein is released.

The entire system of protein folding from translocation to native protein seems to involve two distinct mechanisms of chaperone action. The first is to shield hydrophobic surfaces against aggregation. The second isolates the protein from the rest of the cell and allows folding to proceed to an extent whereby the protein is stable. For instance during translocation or transport across a membrane the first group ensures that the protein does not aggregate whilst crossing the membrane and then it is received by the second

type which aids the protein to refold. These two functions are carried out by the Hsp 70/DnaJ and Hsp60/Hsp10 families respectively.

7.1.3 Stress 90

Stress 90 proteins are the least well understood of the three stress proteins. Like the two other families they are present in all prokaryotic and eukaryotic cells. The various types of Stress 90 proteins found to date share 40% sequence homology (Linquist and Craig (1988)). Like Stress 70 proteins their expression is increased under stress conditions or if the level of unfolded protein in the cell increases. Cytosolic Stress 90 proteins have a molecular mass ranging between 87-92 kDaltons. They associate with a variety of proteins including retroviral transforming proteins, steroid hormone receptors, cellular protein kinases and actin and tubulin. The nature of the interaction of the Stress 90 proteins with the target protein is not well understood. It is believed that the interaction may be due to hydrophobic patches on the surface of the protein ((Hendrick and Hartl (1993)). This is based on the observation that hsp 90 can prevent the aggregation of citrate synthase during refolding (Wiech *et al.* (1992)).

7.1.4 Overview

Molecular chaperones enhance protein refolding by preventing unfavourable intra- and inter- molecular associations from occurring. It is thought that this is achieved by the chaperone binding to the protein and excluding it from the surrounding medium. Although the exact mechanism of binding is unknown, it is thought that due to the non-specific nature of the binding that hydrophobic interactions are strongly involved. As more is learnt about molecular chaperones it is becoming apparent that their is a complex co-operative interaction between the different types of chaperones. It is though that in *in vivo* refolding, stress 70 chaperones bind to the emerging polypeptide during translation. The protein then is passed to stress 60

chaperones and then refold. It is known that protein isomerases are also involved in this process yet their exact role has yet to be elucidated.

In vitro experiments have shown that chaperones can be effective folding enhancers out of the cellular environment. The majority of work to date has been performed with the *E.coli* chaperonins GroEL and GroES and has been aimed at understanding how the chaperone binds to the refolding protein, what structural motifs does the chaperone recognise and what is the release mechanism. These are the biophysical aspect of refolding enhanced by molecular chaperones. No-one has considered the practicalities of using molecular chaperones such as GroEL and GroES on an industrial scale. There is no doubt that the molecules significantly enhance refolding when used at molar concentration equal to the molar concentration of the refolding protein. However, recombinant proteins are expensive and the reusability of the chaperones needs to be assessed if they are to be considered worthwhile when refolding on an industrial scale.

7.2 Materials and Methods

Unless stated otherwise the materials methods used for the GroEL assisted refolding of lysozyme are the same as in Section 3.

7.2.1 Materials

Magnesium adenosine triphosphate, magnesium chloride, tryptone, yeast extract, sodium chloride, phenyl methyl sulphonyl fluoride, ethanol, benzamidine, aprotinin, pepstatin and deoxyribonuclease were obtained from Sigma Chemicals Co., Poole, Dorset. Purification media Sephacryl S-300 was obtained from Pharmacia LKB Biotechnology, Milton Keynes, Bucks. All chemicals were of analytical grade. De-ionised distilled water was used throughout.

7.3 Experimental Methods

7.3.1 Chaperonin Preparation

7.3.1.1 Fermentation

The recombinant strain TG2/pAMI was grown at 30 °C in 16g/dm^3 tryptone, 10g/dm^3 yeast extract, 5 g/dm^3 NaCl and 100 mg/dm^3 ampicillin. A 200 ml portion of an overnight shake culture was used to inoculate 7 dm^3 of growth media in a New Brunswick BioFlo IV fermentor and the cells were incubated at a temperature of 30 °C whilst being stirred at 300 revs/min. Once the optical density at 600nm reached 0.5 units the growth temperature was raised to 42°C and the incubation continued until the cell suspension had reached saturation (Optical Density (O.D.) approximately 3.0 units at 600 nm). Cells were harvested by centrifugation (4000g, 60 minutes) and were used immediately. (See Appendix for growth curve)

7.3.1.2 Purification of GroEL and GroES

All solutions used during the purification of chaperones contained the following unless otherwise stated. 2mM DTT, 2mM EDTA, 0.6 mM phenyl methyl sulphonyl fluoride (previously dissolved in ethanol 5 ml/dm^3 of final

solution), 0.6 mM benzamidine, 1mg/ml aprotinin and 1mg/ml pepstatin. All procedures were performed at 4 °C apart from the fast protein liquid chromatography (FPLC) steps which were carried out at ambient temperature but fractions from these columns were collected on ice. Both the Sephacryl and Q-sepharose purification steps below were performed with the columns attached to a Pharmacia FPLC and the eluate was monitored at 280 nm. The purity of the GroEL was checked at each stage of the purification by SDS electrophoresis using 15% polyacrylamide gels.

Washed cells were re-suspended in 50 mM Tris-HCl buffer, pH 7.5 0.1M NaCl. O.1 g/dm^3 DNAase and 2 mM $MgCl_2$. The cells were lysed by 3 passages through and APV homogeniser (pressure 1000 psig). The cell debris was removed by centrifugation (15,000g , 60 min.). A 20% volume of 6.2% (w/v) streptomycin sulphate solution was added drop-wise to the supernatant which was then stirred for a further 15 minutes. The supernatant was fractionated using ammonium sulphate(NH_4SO_4). Three stages of precipitation were used: 0-10%, 10-30% and 30-60%. The protein precipitating in the final fraction was collected by centrifugation and then dissolved in a minimum volume of 50 mM Tris-HCl buffer pH 7.5, 0.1M NaCl. The solution was fractionated on a column (4.0cm by 60cm) of Sephacryl S300 equilibrated with the above buffer. Fractions (10ml) were collected at a flow-rate of 10ml/min and the presence of GroEL was shown by SDS page. Fractions containing GroEL were combined, concentrated to 10 ml using an Amicon stirred cell ultra-filtration concentrator (3K membrane), and then dialysed into 50 mM Tris-HCl pH 7.5 2mM DTT. Chaperone solutions were then stored at 4 °C.

Figure 7-2 Electrophoresis gel showing the purification of GroEL.

Concentrations of GroES and GroEL were measured using absorbance at 280nm. Extinction coefficients of 3.44 $x10^3$ $M^{-1}cm^{-1}$ and $2.38x10^3$ $M^{-1}cm^{-1}$ respectively were used. These measurements were complimented by measurements using the Coomassie (Biorad) protein assay using bovine carbonic anhydrase as the standard.

7.3.2 Measurement of Protein Concentration

Concentrations of *E.coli* GroEL were evaluated using an absorbance coefficient at A_{280} of $2.38x10^4$ $M^{-1}cm^{-1}$ (Viitanen *et al.* 1990).

7.3.3 Activity Assay

Control experiments were performed using refolding buffer containing 0.1M Tris pH 8.2, 3mM reduced glutathione, 0.3 mM oxidised glutathione, 10 mM ATP, 10mM KCl and 0.007mM GroEL. The addition of refolding buffer to *micrococcus* solution had negligible effect on the absorbance measured at 450 nm.

The effect of the chemicals in the refolding buffer on the assay of the native lysozyme was investigated. 0.1 ml of 10 mg/ml of a solution of native lysozyme in 0.1M Tris HCl pH8.2 was added to refolding buffer and the activity measured. In all cases no aggregation was observed at 450 nm and the activity of native lysozyme in refolding buffer was identical to that in 0.1 M Tris pH8.2.

7.3.4 The effect of the *E.coli* chaperonin GroEL on the refolding of lysozyme.

A solution of 10 mg/ml denatured reduced lysozyme in 0.1M acetic acid was prepared. Different quantities of this solution were added to 10 ml of 0.1M Tris pH 8.2, 3mM reduced glutathione, 0.3 mM oxidised glutathione to give final concentrations of between 0.015 mg/ml and 0.2 mg/ml lysozyme. This was repeated with refolding buffer containing 0.1M Tris pH 8.2 3mM reduced glutathione 0.3 mM oxidised glutathione, 10 mM ATP, 10mM KCl and a concentration of GroEL equal to the final molar concentration of lysozyme in the refolding buffer. For example refolding 0.1 mg/ml lysozyme is the same as refolding 0.007mM lysozyme, therefore the molar concentration of GroEL in the refolding buffer is 0.007mM. The refolding buffer was gently stirred using a magnetic stirrer and all experiments were performed at 25 °C.

7.3.5 Recovery and recycling of GroEL

A solution of 10 mg/ml denatured reduced lysozyme in 0.1M acetic acid was prepared. 0.1ml of this solution were added to 10 ml of 0.1M Tris pH 8.2, 3mM reduced glutathione, 0.3 mM oxidised glutathione, 10 mM ATP, 10mM KCl and 0.007mM GroEL to give a final concentration of 0.1 mg/ml of lysozyme. After 1 hour the activity of the solution was measured.

After refolding, the GroEL was recovered from the refolding buffer by ultrafiltration. The refolding buffer was pipetted into Microsep™ micro-concentrators (Filtron Technology Corporation, Northborough, MA, USA). The molecular cut-off of the membrane was 30,000 Daltons. The micro-

concentrator was spun at 5000 g for 30 minutes in a Centromix centrifuge (Labplant Lab Equipment, Huddersfield, W.Yorks.). The volume of refolding buffer was reduced to 1ml. 9ml of 0.1 M Tris pH 8.2 was added to the concentrated GroEL. This solution was spun as previously explained. The volume was reduced to approximately 1ml. The dilution and concentration was repeated a third time. Each time the concentration step was performed the absorbance and activity of the filtrate was measured. The concentrated sample was diluted with 9ml of 0.1 M Tris pH 8.2, 3mM reduced glutathione, 0.3 mM oxidised glutathione, 10 mM ATP, 10mM KCl. The absorbance and activity of the retentate were measured.

This solution was then used to repeat the refolding experiment. 0.1ml of a solution of 10mg/ml denatured reduced lysozyme in 0.1M acetic acid was added to the solution and the activity measured after one hour. The GroEL was then recovered as previously explained. The procedure of refolding and recovery was performed a total of five times.

7.4 Results and Discussion

7.4.1 The effect of the *E.coli* chaperonin GroEL on the refolding of lysozyme.

As stated previously it has been shown that the aggregation of a number of proteins is inhibited by the prescence of GroEL in the refolding buffer (Viitanen *et al.* (1990), Martin *et al.* (1991) and Mendoza *et al.* (1991)). These studies have involved proteins which are difficult to refold in the absence of molecular chaperones. It is the aim of this section to assess the affect of GroEL on the aggregation and subsequent refolding of lysozyme.

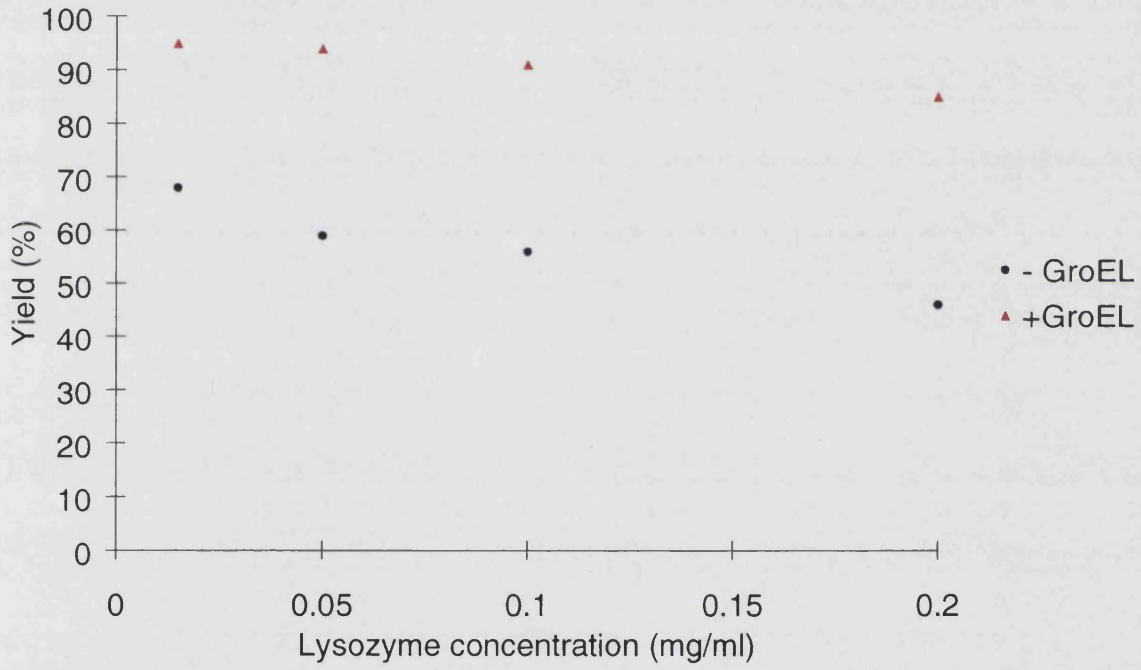

Figure 7-3 The effect of GroEL on the yield of refolding of lysozyme

Figure 7-3 shows clearly that GroEL significantly improves the refolding yield of lysozyme. In the solutions not containing GroEL the yield of refolding gradually decreases from 68% to 46% as the lysozyme concentration is increased from 0.015 mg/ml to 0.2 mg/ml. In the solutions containing GroEL the percentage refolding decreased from 95% to 88% over the same range of

lysozyme concentrations. No aggregates were measured at 450 nm in solutions containing GroEL. In solutions not containing GroEL aggregation increased as the concentration of lysozyme in the refolding buffer increased. The absorbance measured at 450 nm increased from 0 at 0.015 mg/ml lysozyme to 0.8 units at 0.2 mg/ml.

The fact that no aggregates were measured at 450 nm supports the theory that GroEL enhances refolding yields by preventing aggregation. The decrease in yield as the concentration of lysozyme is increased in the experiments with GroEL may well be due to irreversible denaturation during mixing. This theory could be investigated by performing experiments by slowly adding reduced denatured lysozyme using a syringe or micro-peristaltic pump. As the aggregates, if present, are too small to be detected by turbidity measurements, non-denaturing electrophoresis or size exclusion chromatography could be used for analysis.

The results presented here differ from those of Viitanen *et al.* (1990), Martin *et al.* (1991) and Mendoza *et al.* (1991), who found that in the prescence of GroEL and ATP only a small amount of active protein was recovered. In all cases the addition of GroES to the refolding buffer improved the recovery of the native molecule. In contrast to these results the addition of GroES is not necessary for the refolding of dodecameric glutamine synthetase (Fisher (1992))or barnase (Gray and Fersht (1993)). Both the refolding of dodecameric synthetase (Fisher (1992))and barnase (Gray and Fersht (1993)) are improved by the addition of ATP and/or GroES.

There is evidence to suggest that these results are linked to the ease with which the protein refolds. Barnase and dodecameric glutamine synthetase refold spontaneously in the absence of GroEL and GroES. It has been shown for a number of proteins that GroEL binds to some folding intermediate. These include rhodanese (Martin *et al.* (1991)), lactic dehydrogenase (Badcoe *et al.* (1991)) and pre-lactamase (Zahn and Pluckthun, 1992)). It has been

proposed that the binding of these proteins to the GroEL is so strong that unless other co-factors such as ATP and GroES are present the protein will not be released. For the refolding of dihydrofolate reductase (DHFR) it has been shown that release of the molecule from the GroEL complex can be facilitated by the addition of the enzyme substrate to the refolding buffer (Viitanen *et al.* (1991)).

Gray and Fersht (1993) calculated a second order rate constant for the binding of GroEL to barnase. The rate constant was shown to be greater than $1.3x10^{-8}$ $M^{-1}s^{-1}$. This is considerably larger than the rate constant measured for the aggregation of lysozyme. Due to the non-specific nature of GroEL binding and the fact that GroEL has a low affinity for barnase it is unlikely that the binding constant for lysozyme and GroEL will be smaller than that for barnase. In fact as it is thought that hydrophobic interactions are the most important in GroEL binding, it is likely that lysozyme will bind more strongly to GroEL than barnase. Based on the rate constants alone the formation of the GroEL lysozyme complex will occur more quickly than non-productive aggregation. However the mixing time required for the solution to reach homogeneity is in the order of seconds therefore aggregation may well occur prior to GroEL having the chance to interact with refolding intermediates.

It has been shown that GroEL significantly improves the yield of refolded lysozyme. At 0.2 mg/ml lysozyme the refolding yield is increased from 46% to 88%. Using the economic analysis described in Chapter 6 this represents a reduction in cost of $\$3.9x10^6$, from $\$8.8x10^6$ to $\$4.7x10^6$. This is based on identical processes with recycle with identical lysozyme concentrations in the refolding tanks but with different yield. Unfortunately as GroEL is required in equimolar concentrations to lysozyme it is unlikely that the cost of producing the chaperone would justify their use on an industrial scale. Weight for weight the refolding of lysozyme requires approximately 60 times as much GroEL as lysozyme. The process will become more attractive as the

size of the protein being produced increases and if the chaperonin can be recycled cheaply.

7.4.2 The recovery of GroEL

In the previous section it has been shown that GroEL improves the refolding of lysozyme. This leads to a significant reduction in size of the process volume and the process equipment. The process is not likely to be a viable one unless chaperones can be recycled. To assess the applicability of molecular chaperones for use in industrial scale refolding the recovery of GroEL from the refolding buffer has been investigated.

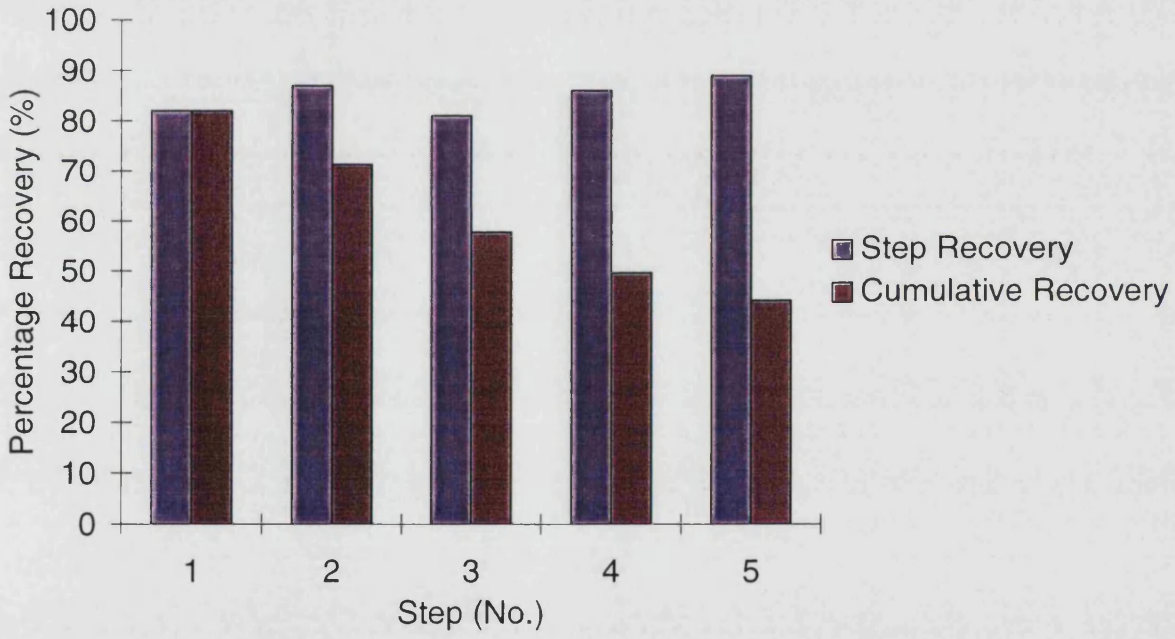

Figure 7-4 The recovery of GroEL using Microsep™ ultrafilters.

Figure 7-4 shows the percentage recovery of GroEL using Microsep™ ultrafilters. The percentage recovery is shown as the actual percentage recovered with respect to the concentration of GroEL in the refolding buffer prior to centrifugation and with respect to the initial concentration of GroEL.

The recovery of GroEL in each separation ranges from 81% to 89%. The total GroEL recovered after five experiments is 44%.

On average 15% of GroEL is lost during the recovery process. The GroEL could bind to the membrane or the sides of the concentrator or could bind to lysozyme denatured on the surface of the membrane. Or the protein could pass through the membrane, however this is highly unlikely as the molecular weight cut-off of the membrane is half the size of the 14 individual subunits of GroEL. Only a few microlitres of fluid are left in the sample collector so losses due to loss of fluid cannot account for the loss of protein. Further studies would have to be carried out to identify exactly where the GroEL is lost.

For the refolding of lysozyme a 15% loss of GroEL during each run would be equivalent to having to produce 6 g of GroEL for each gram of lysozyme. Losses may well be lower if a different type of membrane were used and would be less significant if the target protein were larger than lysozyme. Using membranes may not be the best method for recycling the chaperones. Size exclusion chromatography is often used in the final purification steps of refolding proteins (Datar et al. (1993), Petrides et al. (1995)). Due to the large size of GroEL it could be recovered using this process. Further work is necessary to determine the optimal method for the recovery of the chaperones. Once that is complete an economic study of the viability of using molecular chaperones can be performed.

Approximately 100% of refolded lysozyme is recovered in the filtrate. Approximately 90% was recovered in the first separation. The activity of the refolded lysozyme was measured and compared to the activity of the same concentration of native lysozyme. The refolded protein was 100 % active. In the second step approximately 10% of the lysozyme was recovered. The third sample recovered absorbed only slightly at 280 nm and the activity of the sample was negligible.

7.4.3 The effect of the recovery process on the effectiveness of GroEL as a refolding enhancer

It has been shown that GroEL can be recovered from refolding buffer using ultrafiltration membranes. It is important to investigate the effect of the recovery process on the refolding enhancing properties of GroEL.

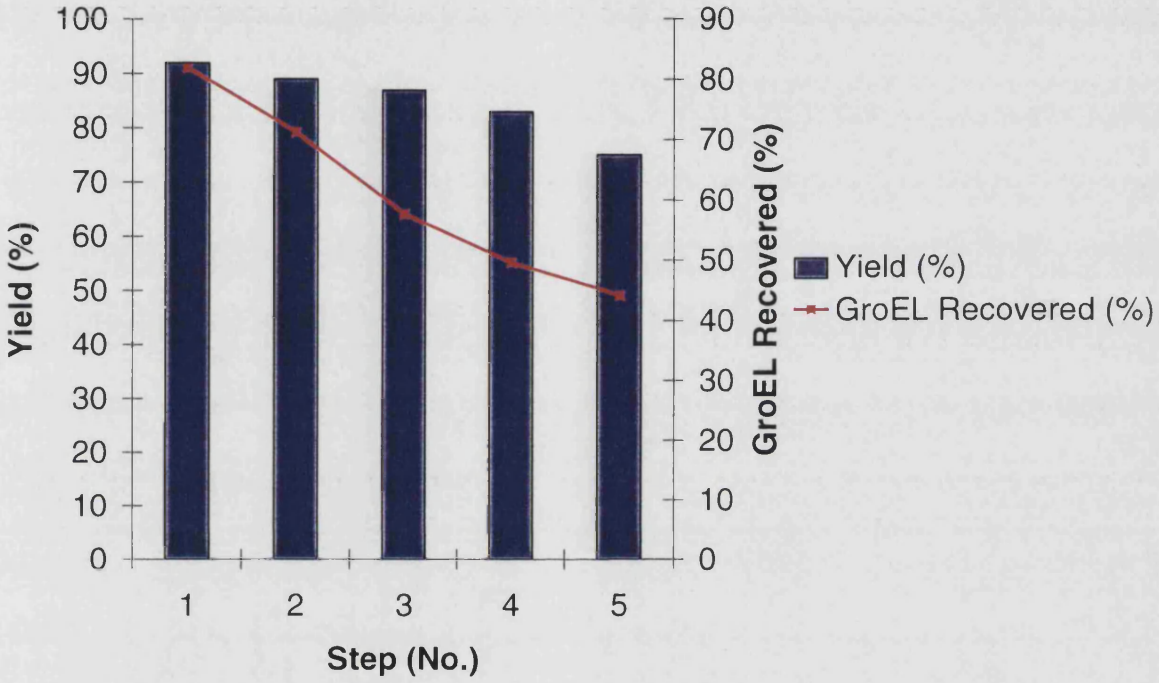

Figure 7-5 The effect of the recovery of GroEL on the refolding yield of lysozyme

Figure 7-5 shows the effect of the recovery of GroEL on the refolding yield of lysozyme. The activity of lysozyme recovered decreases slightly from 92% to 87% in the first three experiments and then falls slightly more to 83% and 75% in the 4th and 5th experiments respectively.

As the yield is only decreased slightly in the first three experiments it is likely that the recovery process has very little effect on the GroEL molecule itself. The drop in yield is probably due to the decrease in the concentration of GroEL. In experiment 4 the molar ratio of lysozyme to GroEL is

approximately 2:1. It has been shown for the refolding of that the optimal molar concentration of GroEL to target protein is greater than 1:1.

7.5 Conclusions

It has been shown that the refolding of lysozyme is significantly enhance by the addition of GroEL to the refolding medium. At 0.2 mg/ml lysozyme the refolding yield is increased from 46% to 88%. As no aggregation was observed in refolding experiments containing GroEL this work supports the theory that GroEL enhances refolding by preventing non-productive aggregation. It has been shown that GroES is not necessary for the release of active lysozyme from the GroEL complex. This supports the theory that proteins that refold spontaneously bind more weakly to GroEL than proteins which readily aggregate even at low protein concentrations.

Using the economic analysis described in Chapter 6 it has been shown that using GroEL to facilitate the refolding of lysozyme that a reduction in cost from $\$8.8x10^6$ to $\$4.7x10^6$ can be achieved. As GroEL is required in equimolar concentrations to lysozyme it is unlikely that the cost of producing the chaperone would justify their use on an industrial scale.

It has been demonstrated that GroEL can be recovered from refolding buffer by the use of ultrafiltration membranes. Using the membranes resulted in a loss of 80% of the GroEL. The reason for the loss is unclear but it is likely to be due to interactions between GroEL and the microconcentrator used for the experiment. There is strong evidence to suggest that the recovery process has no effect on the GroEL molecule and its ability to enhance the refolding of lysozyme. As the concentration of GroEL in the refolding buffer is decreased due to losses associated with the ultrafilters the yield of active lysozyme is decreased this is in accordance with the work of several other researchers.

The use of GroEL as an enhancer for the refolding of small monomeric proteins such as lysozyme is not likely to find economic use. Due to the fact

that the GroEL works most effectively at molar concentrations of 1:1 with respect to the chaperonin and target protein the mass of chaperonin needed to enhance the refolding of small proteins will be prohibitive. If methods of recycling the chaperones are found that are simple and effective chaperones may well find economic use in the production of large proteins which are difficult or even impossible to refold in the absence of chaperones.

8. Conclusions

8.1 The Effect of System Variables on the Batch Refolding of Lysozyme

- The refolding yield of lysozyme is strongly dependant on the solvent conditions of the refolding buffer.

- The optimum pH for the refolding of lysozyme lies between pH8 and pH9. Below pH7 the yield of refolded lysozyme is drastically reduced. This is thought to be due to the chemistry of reacting thiol groups. Under acidic conditions thiol groups on the protein prefer the reduced state thus leading to low refolding yields.

- The concentration of GuHCl and DTT in the refolding buffer have been shown to be important for the refolding of lysozyme. As the concentration of denaturant increases the yield of refolded lysozyme decreases. The concentration of GuHCl at which this occurs is much lower than the concentration of GuHCl needed to inactivate the native molecule. There is evidence to suggest that this is because the sites which are likely to interact with the GuHCl are protected in the native molecule and exposed during in the refolding.

- The yield of refolded lysozyme increases as temperature increases. The yield increases from 23% at 4°C to a maximum of 60% at 50°C. Above 50°C there is a significant reduction in the observed yield. This is likely to be due to thermal denaturation.

- Increasing the concentration of lysozyme in the refolding buffer decreases the yield of refolded lysozyme. The decrease in yield has been shown to be due to a competitive aggregation reaction.

- The method of denaturation affects refolding. Both the type of denaturant and the concentration of lysozyme in the denaturant affect the yield of

refolding. It has been shown that trace amounts of strong denaturants such as urea decrease the yield of refolded lysozyme. Refolding from a weak denaturant, in this case 0.1M acetic acid improves refolding. This is thought to be due to the fact that denatured lysozyme in 0.1 M acetic acid has a more compact structure than in urea or GuHCl.

- When refolding from reduced denatured protein in strong chaotropes the concentration of denatured lysozyme is linked to the concentration of denaturant. The more dilute the protein in the denatured state the more denaturant is transferred to the refolding buffer. Consequently the more dilute the lysozyme the lower the yield of refolded lysozyme.

- If refolding from denatured lysozyme in 0.1M acetic acid the converse is observed. The more dilute the concentration of denatured lysozyme the higher the refolding yield. This is due to reduced interactions between refolding molecules during mixing. The effect is reduced as the concentration of lysozyme in the refolding yield increases. This is because the additional aggregation during the time taken to reach equilibrium becomes less significant when compared to the total aggregation at that concentration.

- The optimum conditions for the refolding of lysozyme are refolding from 1mg/ml reduced denatured lysozyme in 0.1M acetic acid into 0.1M Tris, pH8-9, 50°C with a final concentration of lysozyme of less than 0.15 mg/ml.

8.2 Modelling of the Refolding and Aggregation of Lysozyme

- Lysozyme refolding can be described as a first order reaction. The aggregation of lysozyme has been followed at different concentrations of lysozyme and can be described as a second order process. The apparent rate constant for refolding of lysozyme was found to be 0.147 min^{-1} and the apparent rate constant for aggregation was found to be 3.3 $mgml^{-1}min^{-1}$

- Using these rate constants, a competitive model of refolding versus aggregation has been written. The experimental results agree well with the results predicted by the model. A selectivity term based on the two competitive reactions has been introduced. It has been shown that refolding yields can theoretically be enhanced by stepwise or continuous addition of denatured lysozyme. Denatured reduced lysozyme in 0.1M acetic acid follows the predicted results well.

- Denatured reduced lysozyme in 6M GuHCl and 0.15M DTT does not follow the predicted results well. This has been shown to be due to the increasing concentrations of both guanidine hydrogen chloride and dithiothreitol in the refolding buffer.

- Continuous addition of denatured protein to refolding buffer is likely to be effective for improving the refolding yield of any given protein regardless of the rate of refolding and the rate of aggregation.

8.3 Economic Evaluation of Protein Refolding

- The model was used to investigate the effect of protein concentration and yield in the refolding tanks on the cost of the entire refolding process. Using the total equipment purchase cost as a benchmark it has been shown that for the refolding of lysozyme an optimal refolding concentration 0f 0.22 mg/ml exists.

- For the refolding of lysozyme the two most important costs have been shown to be ultrafiltration and fermentation.

- It has been shown theoretically that the total purchase cost and subsequently the production cost of a refolding process can be reduced significantly by recycling aggregated material from the refolding tanks to the solubilisation tanks.

8.4 Chaperone Assisted Refolding of Lysozyme

- Refolding was enhanced by the use of the *E.coli.* molecular chaperone, GroEL. GroEL significantly improves the yield of refolded lysozyme in the range 0.015 mg/ml to 0.2 mg/ml.

- The recovery and effectiveness thereafter of GroEL has been studied. GroEL was recovered (>80%) using ultrafiltration. It has been shown that after five refolding experiments the effectiveness of GroEL as a refolding enhancer is unaffected by the recovery process.

8.5 Recommendations for Future Work.

8.5.1 Refolding on a larger scale.

It has been shown that the refolding of lysozyme is highly dependant on the concentration of denatured reduced lysozyme prior to refolding. To maximise the yield of refolded protein the refolding buffer should attain homogeneity as rapidly as possible. This has significant implications for refolding on an industrial scale. To date studies of refolding have been performed on a small scale, generally involving millilitres of refolding buffer. Large scale refolding experiments, i.e. refolding volume greater than $1m^3$, should be performed. The effect of having a number of addition points, the placement of addition points, the concentration of the denatured protein, Reynolds number and mixing regime on the refolding yield need to be investigated.

8.5.2 Aggregation Studies

The aggregation of refolding intermediates has been observed. It has been shown that aggregation increases as the concentration of lysozyme in the refolding buffer increases. It has also been shown that the aggregation of lysozyme can be described by a second order reaction with respect to protein concentration. The effect of other environmental parameters such as temperature, pH, etc. on aggregation should be investigated.

To improve the understanding of the specific mechanism of aggregation further experimental studies should be performed. Stopped-flow fluorimetry techniques could be used to investigate the kinetics of aggregation at low protein concentration. The size of aggregates formed over time could be identified using non-denaturing gel electrophoresis and size exclusion chromatography. It has been suggested that aggregation occurs between specific structural motifs of refolding intermediates (Seckler *et al.* (1989)). This could be investigated using site directed mutagenesis. By changing a site and seeing if it has an effect on the aggregation process the theory could

be studied.. This data could then be used to propose an accurate model of aggregation during refolding.

8.5.3 Step-wise and Continuous Addition.

Refolding lysozyme by step-wise or continuous addition of denatured lysozyme to the refolding buffer improves refolding yields significantly. This works needs to be repeated using different proteins to see if they too can be modelled using this method. If the apparent rate constants for different proteins can be established the effectiveness of the continuous and step-wise addition of the protein can be assessed using the model proposed here. It is my belief that this method of refolding enhancement is generically applicable to all proteins as long as significant changes in the refolding buffer solvent conditions can be avoided.

The continuous addition of lysozyme has been shown to follow the predicted model well at low concentrations. As the rate of addition is increased an anomaly is observed, rather than seeing a decrease in the yield the yield increases. It is possible that this is due to the increasing viscosity of the solution and the increase in concentration of native protein which reduces interactions between folding intermediates. This process of continuous addition needs to be studied further.

The effect of the continuos addition of protein on the cost of refolding needs to be modelled. An optimum rate of addition will exist depending on the rate constants for aggregation of the specific protein being studied.

8.5.4 Centrifugal Recovery and Refolding of Protein Refolding Aggregates

Is has been shown that by recovering refolding aggregates it is theoretically possible to reduce the costs involved with the refolding of lysozyme. This practice would improve the refolding of any protein. The recover of refolding aggregates needs to be studied. Refolding aggregates, unlike protein crystals

are soft and should be stable in high shear fields. The recovery of the aggregates could be performed by centrifugation or by using hydrocyclones. The work needs to be performed with a variety of proteins.

8.5.5 Applicability of GroE Assisted Refolding on an Industrial Scale

It has been shown that GroEL can be easily recovered from refolding buffer by ultrafiltration and that the ultrafiltration process has no effect on the refolding enhancing properties of GroEL. The separation of GroEL from lysozyme is facile due to the large difference in size of the two molecules. Also GroES is often needed to facilitate the release of proteins bound to GroEL. As such it is important to investigate other methods of recovery. This could involve some form of chromatography, e.g. size-exclusion, ion exchange or affinity chromatography. As these processes are already present in the purification of the target protein the additional cost of recycling the chaperones should be minimal. The cost of producing the chaperones and the additional cost of ATP may however be prohibitive for their use on industrial scale.

8.5.6 Novel Reactor Conformation

The ideal refolding system would involve the instantaneous dilution of the protein to be refolded to the optimal conditions of refolding. Mass transfer of the protein in the refolding buffer should then be limited as much as possible. This is almost impossible in a stirred tank. It may be possible using an ejector system. The denatured protein is added in to the ejector where the venturi effect causes a high degree of turbulence. The turbulence is then rapidly converted to streamline flow.

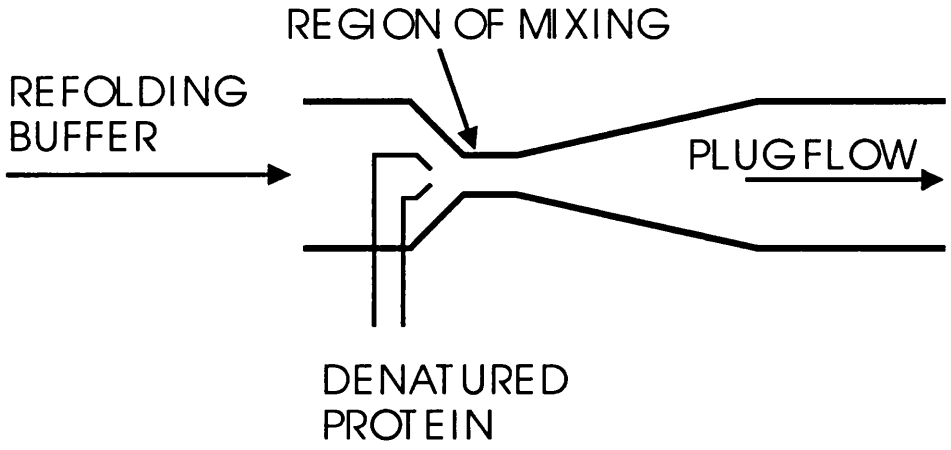

Figure 8-1 Ejector system for refolding

Plug-flow may be difficult to achieve, if some form of polymer was placed in the section of the ejector downstream of the mixing zone this might improve its performance.

9. Nomenclature

k	Specific rate constant (s^{-1}) or general rate constant ($f\{\psi\}$)
R	Gas constant (J/mol K)
k_O	Frequency factor (s^{-1})
T	Temperature (K)
E	Activation energy (J/mol)
r_A	Rate of refolding (mg/ml.s)
X_D	Conversion of denatured protein
C_0	Initial concentration of denatured protein (mg/ml)
C_D	Concentration of denatured protein at time t (mg/ml)
C_N	Concentration of native protein (mg/ml)
k_1	First order rate constant (min^{-1})
t	Time (min or s).
C_Y	Concentration of product (mg/ml)
C_X	Concentration of reactant (mg/ml)
ψ	General order of reaction
k_2	Second order rate constant (mg/mg min)
A_{FC}	Fixed capital cost ($)
A_{CON}	Annual cost of consumables ($)
A_{UT}	Utilities and labour costs ($)
A_U	Cost of the ultrafiltration unit ($)

a_M	Area of membrane (m^2)
a_C	Cost of the membrane per unit area ($/$m^2$)
J	Flux (l/m^2h)
A_{C2}	Cost of a centrifuge ($)
Σ	Settling capacity (m^2)
Q	Flow-rate (l/h)
V_R	Volume of the refolding tanks (m^3)
A_R	Cost of the refolding tanks ($)
V_S	Size of solubilisation tanks (m^3)
A_S	Cost of the solubilisation tanks ($)
C_S	Concentration of denatured protein in solubilisation tanks (g/l)
A_H	Cost of the fermenter ($)
V_F	Volume of the fermenter (m^3)
α	Cost capacity exponent
β	Cost capacity exponent

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11. Appendices

11.1 Lysozyme

Hen egg-white lysozyme is one of the most extensively studies enzymes and was the first enzyme to have its three dimensional structure determine by X-ray crystallography. The primary structure was determined by Jolles *et al.* (1963). It is a single sub-unit protein containing 129 residues with a molecular weight of 14,000. Lysozyme is an extremely stable enzyme and is cross linked by four di-sulphide bonds. Theses bonds are between residues 6-127, 30-115, 64-80, 79-64.

Blake *et al.* (1965) determined the three dimensional structure of lysozyme. The lysozyme molecule is small, compact and approximately spheroid with dimensions of 45/30/30 angstroms. There are two structural domains which are divided by a deep cleft. The active site is formed by the residues which form the link between the two clefts. One structural domain contains the N- and C- termini of the protein whilst the other domain contains the central region of the polypeptide chain.

Lysozyme contains approximately 40% α-helix and a small amount of anti-parallel β-sheet. There are four regions of helical structure. Residues 4-15, 24-36, 88-96 and 108-115. Although slightly distorted these structures contain approximately 3.6 residues per turn. The only region of β-sheet is formed by residues 42-60. Apart from an S-shape alignment between residues 55-60 and 50-54 the rest of the structure is mainly aperiodic.

In its native form, the majority of the surface of lysozyme is hydrophilic, with three only three tryptophan residues exposed. The majority of the hydrophobic residues are contained in the core of the protein and are surrounded by polar residues.

Lysozyme cleaves bacterial cell walls and chitin. It hydrolyses the β1-4 glycosidic bond between N-acetylglucosamine (NAG) and N-acetylmuramate (NAM). The active site of lysozyme can accommodate six units of polymeric substrate. The critical residues in the active site are Asp 52 and Glu 35. The action of the cleavage is as follows:

The substrate binds to lysozyme in the active site and is held in place by hydrogen bonds and van der Waals forces. During this process the ring of the fourth (D) sugar residue becomes distorted. A proton is transferred from Glu 35 to the oxygen in the glycosidic bond. This results in the cleavage of the bond. This creates a positively charged carbonium ion which is stabilised by the negatively charged Asp 52 until it can react with a hydroxyl group from the solvent

Loss of enzyme activity due to the chemical modification of either Asp 52 or Glu 35 has shown that these amino acids are essential for the catalytic action of the enzyme. (Yamada *et al.* 1972). Mono-, di-, and trisaccharides have been shown to inhibit lysozyme. They compete for positions on the active site of the protein.

Egg-white lysozyme is produced in the tubular glands of the oviduct of chicken and is regulated by steroid hormones.

The denaturation of lysozyme has been studied by several groups. To follow the denaturation of a protein it is important to measure a property which reflects the conformational state of the protein. Activity is very sensitive to changes in solvent conditions. Loss of activity an occur due to very small changes in the shape of the active site and does not give a great deal of information about the structure of the protein. Loss of activity is almost always a precursor to denaturation. To measure large structural changes the following techniques have been used; difference spectroscopy, circular dichroism, fluorescence, viscometry, 1-anilino naphthalene sulphonate (ANS)

binding, hydrogen exchange labelling and nuclear magnetic resonance and mass spectrometry. These have been reviewed and discussed in detail in a review by Dobson *et al.* (1994).

The native structure of lysozyme is very stable to extremes of pH at room temperature. Measurements of optical properties showed no significant change in conformation over the pH range 1.3-11.3 in dilute salt concentrations (Imoto *et al.* (1972)). Lysozyme is also extremely stable with respect to high temperature. At neutral pH significant structural change does not occur until 75 °C. As with most proteins the effects of pH and temperature with respect to denaturation have been shown to be cumulative. Sophianopolous and Weiss showed that at pH 3 the transition temperature for thermal denaturation of lysozyme fell to 45 °C.

The effect of chemical denaturants on the structure of lysozyme have also been investigated. It was found that at neutral pH and 25°C that 9 M urea had little effect on either the tertiary or secondary structure of lysozyme. However under these conditions if the pH was reduced to below 4 or the temperature was raised above 35°C then denaturation did occur. Similar results were obtained by adding a thiol reducing agent to the system.

Studies of the denaturation of lysozyme in GuHCl have shown that little structural change occurs below 3M GuHCl. At concentrations above this the molecule starts to be denatured and is fully denatured at 5M GuHCl. No further structural changes could be induce by increasing the denaturant concentration or decreasing the pH or increasing the temperature. Aune *et al.* have shown that all (in their study)proteins are fully denatured in 6M GuHCl. They also showed that lysozyme denatured at low pH and high temperature can be further denatured by the addition of 6M GuHCl. Like for urea the denaturing effect of GuHCl is temperature dependant. The transition concentration for GuHCl at 25 °C is 3M however at 45°C the

transition occurs at 2M GuHCl (Tanford *et al.* (1966)). The results show that GuHCl is a more effective denaturant the urea (Imoto *et al.* (1972)).

The prescence of di-sulphide bonds in lysozyme makes the molecule inherently stable. The molecule is fully unfolded in 6M GuHCl. This is to say there is no observable secondary structure in lysozyme dissolved in 6m GuHCl. However upon addition of reducing agents the hydrodynamic volume of the denatured molecule increases.

The refolding of non reduced and reduced lysozyme has been the subject of several studies. The method of refolding has generally been to reduce the concentration of denaturant and reducing agent by dilution into a refolding buffer.

The refolding of non reduced lysozyme is facile and yields of 90% at concentrations greater than 0.5 mg/ml are possible (Tanford *et al.* (1966)). Refolding of non-reduced lysozyme has been studied by circular dichroism. These studies have shown that that 80 % of the native structure is regained in less than 4 ms (Kuwaujima *et al.* (1985), Ikeguchi *et al.* (1986), Chaffotte *et al.* (1992)).

The refolding of reduced lysozyme is more complicated and involves the oxidation of the four di-sulphide bonds.

Epstein and Goldberger (1963) studied the refolding of reduced denatured lysozyme. They showed that refolding was dependent on the protein concentration, pH, thiol concentration and temperature. They showed that the yield of refolded protein increased from pH 7- pH 8.5 and that a higher yield was achieved at 38°C than at 25°C. Saxena and Wetlaufer (1970) performed a similar study and found that the optimal conditions for refolding with respect to thiol concentration were 5 mM reduced glutathione and 0.5 mM oxidised glutathione. Some years later Goldberg et al. (1991) performed

a similar study of refolding of reduced denatured lysozyme. They obtained a much lower yield than preciously reported despite very similar refolding conditions to those used by Saxena and Wetlaufer.

Like for the refolding of reduced lysozyme it has been shown that 80% of the secondary structure of reduced denatured lysozyme returns after 4-10 ms (Chaffotte *et al.* (1992)). A comparative study of the refolding of lysozyme via air-oxidation and thiol di-sulphide interchange was performed by Perraudin *et al.* (1983). Their work supported the work of Saxena and Wetlaufer (1970) and showed that refolding was more rapid and achieved higher yields via the thiol route. This is thought to be due to the fact that incorrectly formed di-sulphide bonds reforming correctly is the rate limiting step in the refolding of reduced denatured lysozyme.

11.2 Techniques for studying refolding

These methods are numerous and were first summarised by Creighton in 1978. A more recent review by Dobson *et al.* (1994) gives a comprehensive account of present technology. Techniques available for studying refolding include:-

Activity measurements.
Possibly the only sure way of measuring the extent to which the protein has refolded to the native state is to measure the activity directly. Unfortunately these assays are often difficult and time consuming which means that they are very difficult to apply to kinetic studies. Also when measuring activity it is usually reported in terms of percentage of native activity. These assays give no indication of whether the refolded protein is all 30% active or 30% of the protein is fully active.

Far U.V. Circular Dichroism (C.D.)
The far U.V. spectrum measures the extent of secondary structure in a protein. Stopped flow techniques have been used successfully to determine the rate constants for the refolding of several proteins. Care must be taken in applying these constants to any form of kinetic model as even though a protein refolds to a state where it has reformed 99% of its secondary structure it may still be inactive and tertiary structure assembly is usually more important from a process point of view.

Near U.V. Circular Dichroism.
The near U.V. spectrum measures the extent of tertiary structure in a protein. Near U.V. C.D measures changes in the absorbance of aromatic residues as they are immobilised in the tertiary structure of the refolding protein. Near U.V. C.D. is sometimes more useful than far U.V. C.D. when the protein in question has a high degree of aromatic residues.

Fluorimetry.

Measures the physical state of tryptophan (and tyrosine) residues. tryptophan residues absorb light at 275-295 nm and fluoresce at 330-340 nm. A good technique for measuring extent of refolding and approximately 100 more sensitive than spectrophotometry. However as for C.D. measurements identical fluorimetry traces for native and refolded protein do not guarantee that the refolded protein is active.

ANS binding. 1-anilino naphthalene sulphonate binds to exposed hydrophobic surfaces allowing the extent of refolding to be measured.

Inhibitor binding.

Measures the formation of the native state. This method must be used carefully as some inhibitors will bind to the so called molten globule state of the protein. Not as reliable a technique for identification of the native state as activity measurements.

Hydrogen exchange labelling and nuclear magnetic resonance.
Measures the formation of persistent hydrogen bonds and their burial from solvents. Used extensively to measure very rapid reactions.

Hydrogen exchange labelling and Electron Spin Mass Spectrophotometry (ESMS). Detection of transient intermediates and folding populations; especially in the early stages of refolding.

Light scattering.

Aggregation can be monitored by measuring the light scattering properties of aggregates as they "drop out" of solution. This can be achieved by measuring absorbance outside the absorbance spectra of the protein in question.

Which technique is used will depend on availability, ease of use, ease of analysis and the properties of the particular protein being studied. As the refolding of lysozyme is relatively slow (~ 1 hour to completion) and its activity assay is simple, the assay can be used for monitoring the kinetics and extent of refolding. For a more detailed description of the assay see Materials and Methods. As aggregation is much more rapid stopped-flow spectrophotometry is used.

11.3 The effect of the type of denaturant on the rate of refolding of lysozyme

Various quantities of 10 mg/ml denatured reduced lysozyme in 6M GuHCl and 0.15M DTT were added to 200 ml of refolding buffer at 40 °C to give the desired final concentration of lysozyme. The recovery of activity over time was measured using the assay described in section 3.3.2. The extent of aggregation was measured at 450 nm in a spectrophotometer as described in section 3.3.4

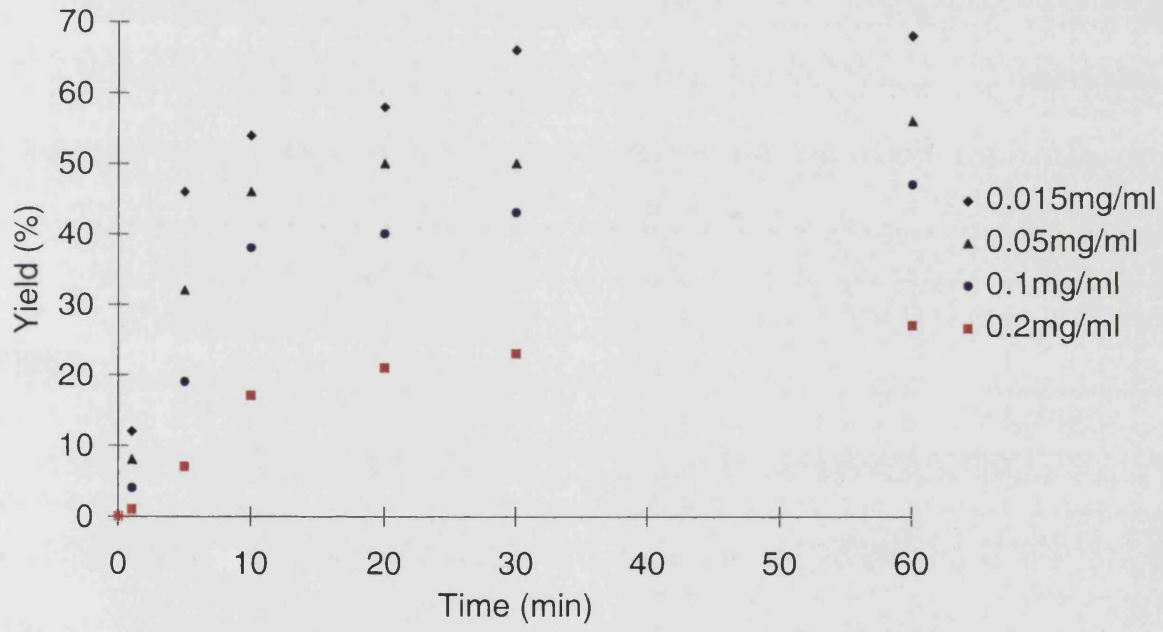

Figure 11-1: The effect of final protein concentration on the yield of refolded lysozyme denatured in GuHCl.

The maximum refolding yield (68%) was obtained at 0.015 mg/ml. The yield decreased as the concentration of lysozyme in the refolding buffer increased.

Various quantities of 10 mg/ml denatured reduced lysozyme in 8M urea and 0.15M DTT were added to 200 ml of refolding buffer at 40 °C to give the

desired final concentration of lysozyme. The recovery of activity over time was measured using the assay described in section 3.3.2. The extent of aggregation was measured at 450 nm in a spectrophotometer as described in section 3.3.4

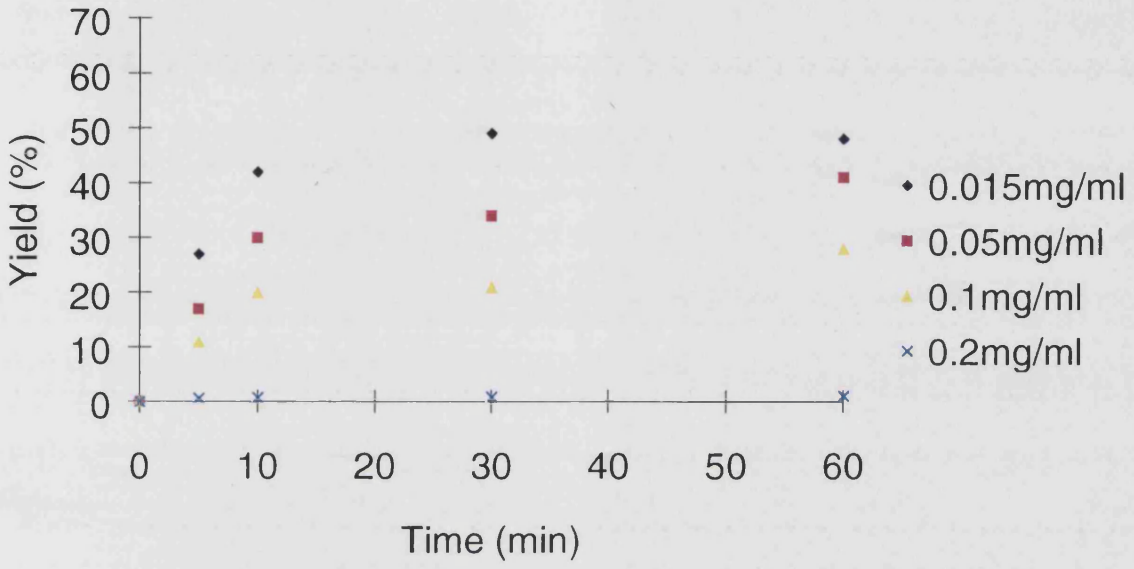

Figure 11-2 The effect of final protein concentration on the yield of refolded lysozyme denatured in urea.

Various quantities of 10 mg/ml denatured reduced lysozyme in 0.1 M acetic acid were added to 200 ml of refolding buffer at 40 °C to give the desired final concentration of lysozyme. The recovery of activity over time was measured using the assay described in section 3.3.2. The extent of aggregation was measured at 450 nm in a spectrophotometer as described in section 3.3.4

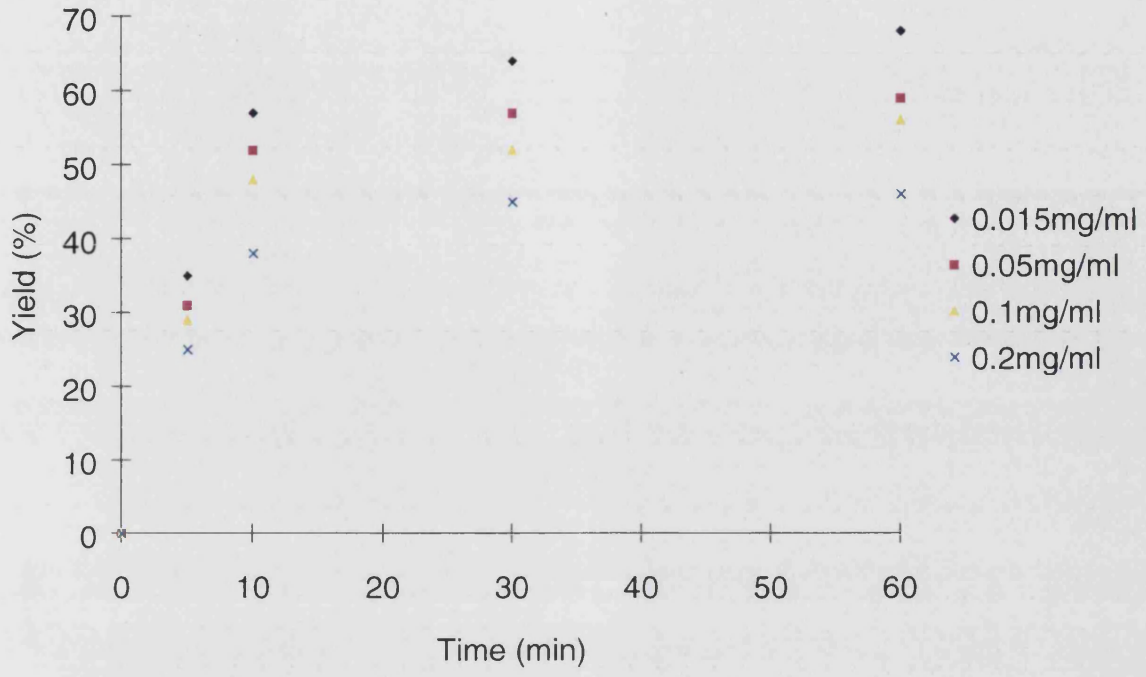

Figure 11-3 The effect of final protein concentration on the yield of refolded lysozyme denatured in acetic acid.

11.4 The inactivation of lysozyme in GuHCl and NaCl

Lysozyme (0.2 mg/ml) was inactivated in 0.06 M potassium phosphate buffer pH 6.2 in the prescence of increasing concentrations of GuHCl and NaCl at 20°C for 15 minutes. The enzyme activity was them measured. The relative activity was reported as a percentage of he activity of the same concentration of lysozyme in the absence of GuHCl or NaCl.

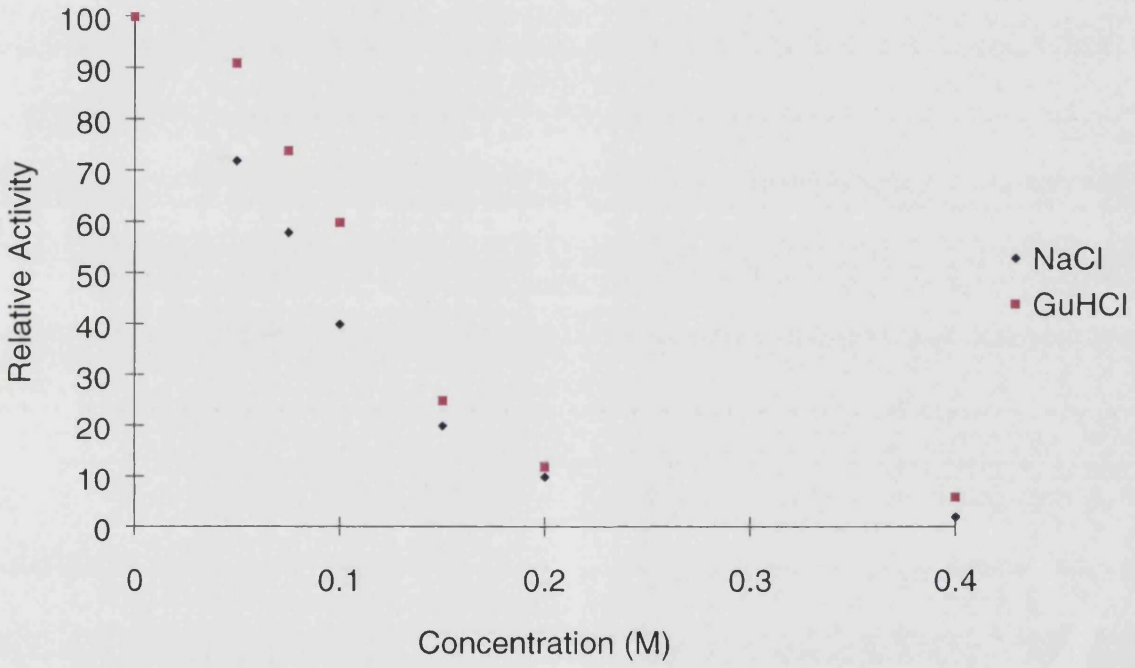

Figure 11-4 The effect of GuHCl and NaCl on the activity of lysozyme.

Both NaCl and GuHCl affect the relative activity of lysozyme in the same manner. This suggests that the inactivation of lysozyme by GuHCl is due to its ionic nature. This explains the difference observed between inactivation of lysozyme and urea. Much higher concentrations of urea are necessary to inactivate lysozyme (West *et al.* (1996)).